ENCYSTMENT STAGES OF DICTYOSTELIUM [1]

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The genus *Dictyostelium* Brefeld (1869) is representative of the Acrasieae, a group of simple, cellular slime molds, wherein the life cycle consists of an amoeboid vegetative phase and a plant-like fruiting phase. The vegetative phase of these primitive micro-organisms is characterized by the independent movement and multiplication of free-living myxamoebae which feed by the ingestion and digestion of bacterial cells. The fruiting phase normally begins with the exhaustion of this food supply and is first evidenced by the coordinated inflowing of the myxamoebae to form wheel-shaped aggregates, or *pseudoplasmodia.* It attains its definitive expression as a portion of the myxamoebae thus assembled becomes transformed into vacuolate, parenchyma-like cells to form the upright stalk, or *sorophore,* whilst the remainder differentiate into capsule-shaped reproductive cells, or *spores,* to form the elevated spore mass, or *sorus,* of the slime mold fructification, or *sorocarp.* In most cultures of *Dictyostelium* grown under favorable cultural conditions, virtually all of the former vegetative myxamoebae enter directly into radiate pseudoplasmodia and subsequently differentiate into either stalk cells or spores (Olive, 1902; Raper, 1935, 1940a, 1940b; Bonner, 1944). However, marked exceptions to this behavioral pattern occur in certain species and strains.

Under some conditions, not wholly understood, many of the vegetative myxamoebae never enter the fruiting state (*i.e.,* aggregate to form pseudoplasmodia) but as individual cells enter an encystment stage. Such resting cells are termed *microcysts* (Fig. 1). This designation was first applied by Cienkowski (*fide* Olive, 1902) to describe those myxamoebae of *Guttulina* which under unfavorable conditions tended to form rounded protoplasmic bodies with definite ectoplasmic membranes. Olive widened the application to include other Acrasieae and stated that microcyst formation occurred under unfavorable conditions such as slow drying in hanging-drop preparations. In our investigations such individually encysted cells have been observed from time to time and in large numbers in agar plate cultures of *Dictyostelium mucoroides* Brefeld (1869), and with even greater frequency in *D. minutum* Raper (1941), *D. polycephalum* Raper (1956a, 1956b), *Polysphondylium pallidum* Olive (1901, 1902), and *Acytostelium leptosomum* Raper (1956b). They are believed to occur in greater or lesser numbers in all aging cultures.

An additional, multicellular encystment stage occurs in occasional strains of *Dictyostelium mucoroides* and in many isolates of *D. minutum.* These relatively complex structures arise by a morphogenetic process possibly alternative to normal

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sorocarp formation. Because of their larger dimensions and their multicellular origin and constitution, these bodies are termed *macrocysts* (Fig. 2). This designation was first applied by Raper in 1951 with reference to structures seen in certain cultures of *D. minutum* isolated from soil, but no description of the macrocysts was given. More recently, Cormier and Raper (1955) and Raper (1956b) have reported briefly concerning their formation and structure. It is of special interest that Brefeld, in his original paper on *D. mucoroides* (1869), described and illustrated as "dwarfed sporangia" structures which are believed to have represented macrocysts (Fig. 3).

The primary purpose of this investigation has been to extend our observations on these long neglected structures and to seek answers to three basic questions pertaining to them, namely: How are the macrocysts and microcysts formed? What conditions favor their development? What are the roles of these encystment stages in the life cycle of the Acrasieae? A resumé of our present knowledge of these matters is presented herewith.

Materials and Methods

Micro-organisms investigated

Several cultures of *Dictyostelium* were examined relative to the production and possible function of the macrocysts, including *Dictyostelium mucoroides* (Strains S–28b, NC–12, and WS–47) and *D. minutum* (Pur–8a and WS–56–2). *Dictyostelium mucoroides* was the slime mold studied most intensively, particularly strain S–28b which produces abundant macrocysts. Macrocysts were first observed and photographed in strain NC–12 when this slime mold was isolated in 1937 (Fig. 2).

Microcysts of several species of the Acrasieae were studied, including those of *Dictyostelium minutum* (WS–116b), *D. polycephalum* (S–4), *Polysphondylium pallidum* (WS–116c), and *Acytostelium leptosomum* (FG–12a). Those of *D. polycephalum* are illustrated in Figure 1.

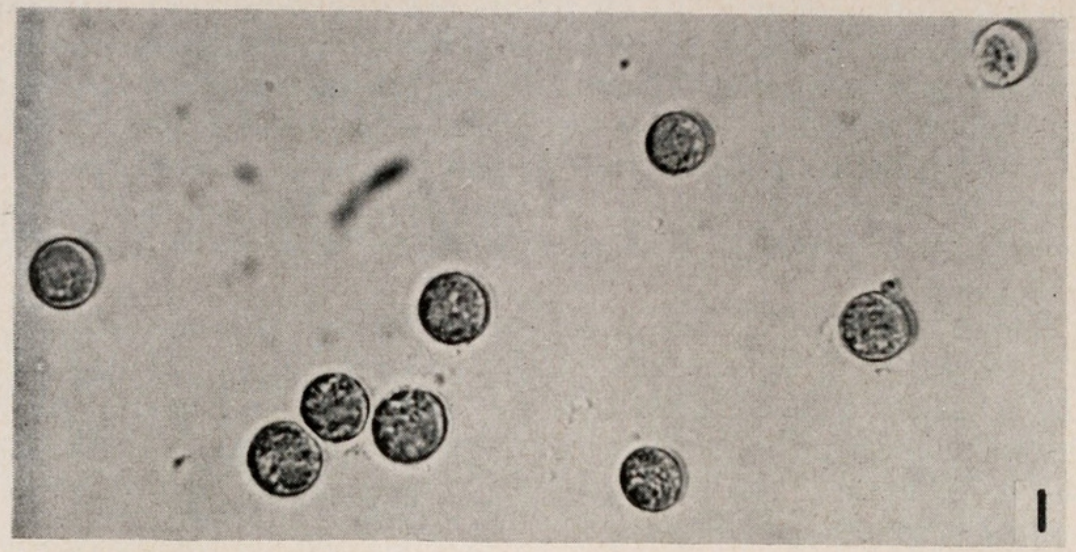
Different bacterial associates were investigated as food sources for the slime molds and for their possible influence upon macrocyst formation. Included among gram-negative species were *Escherichia coli* (No. B–281), *Aerobacter* sp. (Singh's strain), *Aerobacter aerogenes* (Sussman's strain), *Flavobacterium* sp. (DIF), *Serratia marcescens* (No. B–175), and *Pseudomonas fluorescens* (No. B–112); included among gram-positive species were *Bacillus megaterium* (No. B–160), *B. subtilis* (Sarles' strain), and *Sarcina lutea* (No. B–1018). *Escherichia coli,* the organism used most commonly, is a short rod which is mildly proteolytic and ferments both dextrose and lactose; the myxamoebae appeared to grow best when feeding upon this bacterium.

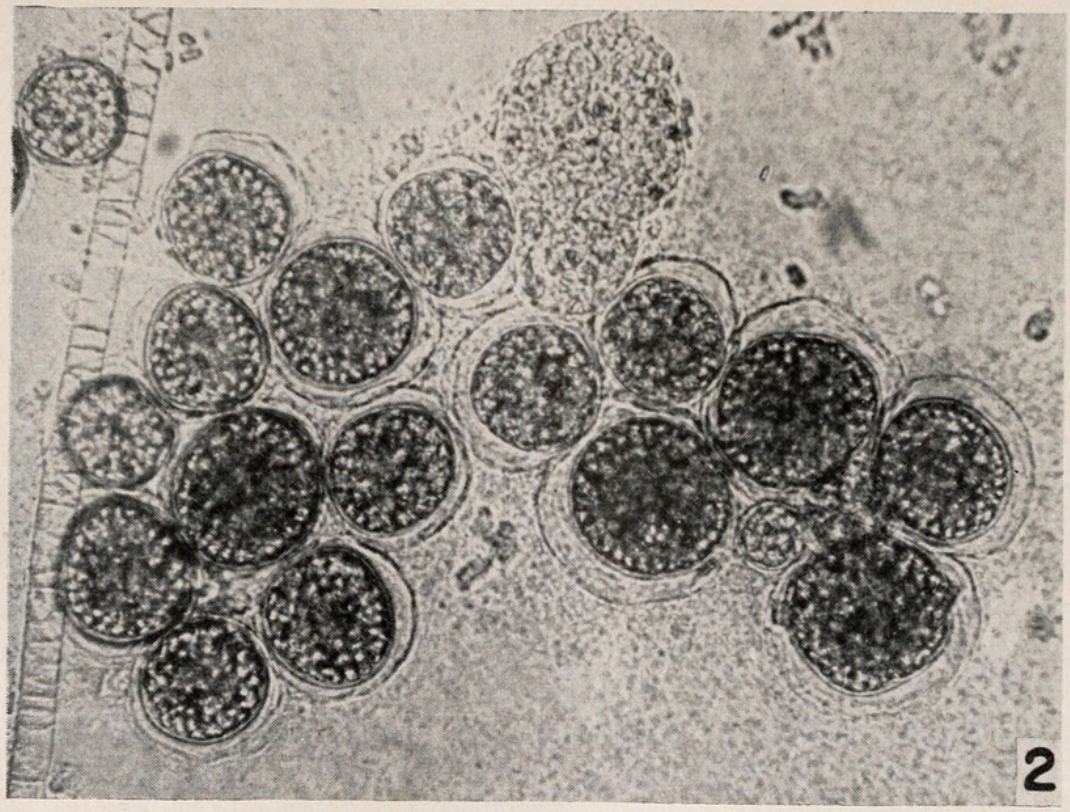
Cultivation of the slime molds

Environmental and cultural conditions, including (1) temperature, (2) culture media, (3) pH of substrate, (4) ammonium ion concentration, and (5) per cent relative humidity, were varied to determine their effect upon macrocyst formation in *Dictyostelium.*

Incubation temperatures used in most of the experiments were 10, 15, 20, 25,

PLATE I





and 30° C. The optimum growth temperature for the macrocyst-forming strains of *Dictyostelium mucoroides* was about 20° C., whereas that of the *D. minutum* isolates more nearly approximated 25° C.

Many different media were employed for the cultivation of the slime molds and the associated nutritive bacteria, which, for the most part, were patterned after substrates previously reported by Raper (1951). An agar medium containing 0.1% lactose and 0.1% peptone (0.1 L–P agar) was used most extensively since it provided the most reproducible growth and the most consistent macrocyst formation in *D. mucoroides* and in *D. minutum*. Horse dung-infusion and 0.05% uric acid agars [3] were also employed because of Brefeld's report (1869) that dung extract and uric acid induced spore germination.

The pH of the substrates employed for the conjoint growth of the slime molds and associated bacteria was varied by buffering with KCl, potassium acid phthalate, KH_2PO_4, and K_3BO_3 as recommended by Clark and Lubs (Clark, 1928). Bacteria were also pre-grown on buffered 0.1 L–P medium of varied pH and transferred to unbuffered medium prior to inoculation with the slime mold (Raper, 1951). Experiments to determine the possible effect of ammonium ions on growth and macrocyst formation in *Dictyostelium* were patterned after those reported by Cohen (1953).

Different relative humidities were obtained by placing specified concentrations of H_2SO_4 in water in desiccators, as reported by Wilson (1921). Two types of slide cultures were employed: (1) Maximov tissue culture slides containing one ml. 0.1 L–P agar inoculated with a mixed suspension of spores, or myxamoebae and macrocysts, of *D. mucoroides* and *E. coli* cells; (2) plain flat slides spread with 1.0 ml. molten 0.1 L–P agar and inoculated by on-flowing a mixed suspension of slime mold and bacteria. The latter slides were supported in an upright position by small wooden blocks during incubation.

Agar plate cultures were grown routinely in glass Petri dishes, to maintain a high per cent relative humidity, and incubated in the dark at varying temperatures. The plates were cross-streaked or completely smeared with the bacterial associate and then inoculated at the center with spores, macrocysts, or myxamoebae of the selected slime mold, the type of inoculum and bacterial associate being varied with the experiment.

[3] Dung decoction was made by autoclaving 100 grams of fresh horse dung/liter of water for twenty minutes at 15 pounds' pressure. The resulting decoction was filtered, solidified with 1.5% agar, and re-sterilized (final pH 6.1–6.35). Uric acid agar consisted of a 0.05% aqueous solution of uric acid to which 2.0% agar was added.

PLATE I

FIGURE 1. Microcysts of *Dictyostelium polycephalum*, representing individually encysted vegetative myxamoebae. × 530.

FIGURE 2. Macrocysts of *Dictyostelium mucoroides*, Strain MC–12, showing the characteristic habit and structure of these bodies; the cellular stalk of a normal sorocarp appears at the far left. × 250.

FIGURE 3. Illustrations from Brefeld's description of *Dictyostelium mucoroides* (1869), from left to right: "Dwarfed sporangia," which are believed to be identical with the macrocysts reported in this paper; "small sporangia" with "rudimentary stalks" in the spore-forming plasm; "sporangium" with a small stalk surrounded by an enclosing membrane. × 300.

Reagents

Several reagents were employed to establish the cellulosic nature of the walls of the macrocysts, namely:

(1) Chloroiodide of zinc solution was prepared by dissolving 30 grams of ZnCl, 5 grams of KI, and 0.89 gram of iodine in 14 ml. of distilled water (Stevens, 1916). The material to be tested was mounted in water, after which the reagent was applied to one edge of the cover glass and drawn under it by placing a piece of filter paper against the opposite edge. Cellulosic material stains violet-blue.

(2) Congo red was prepared as a 0.5% aqueous solution of the dye made alkaline by adding two or three drops of concentrated NaOH. In an alkaline solution this reagent stains cellulose red, which when put in HCl turns blue (Raper and Fennell, 1952). This is a presumptive but non-specific test for cellulose.

(3) Schweitzer's reagent was prepared by bubbling air through 60 ml. of NH_4OH containing 10 grams of fine copper turnings for one hour (Hodgman, 1951). Cellulose is quickly dissolved by this strong cuprammonium solution. In practice, the macrocysts were generally mounted in water and the reagent was applied by drawing it under the cover glass, hence diluting and advantageously slowing its action. A 72% aqueous solution of H_2SO_4 was also used as a cellulose solvent.

(4) A strong birefringence in polarized light was likewise interpreted as confirming the predominantly cellulosic composition of the macrocyst wall.

Nile blue sulfate was employed as a vital dye for staining myxamoebae and progressive developmental stages as reported by Bonner (1952). It was also incorporated into 0.1 L–P agar prior to inoculation with *E. coli* and the slime mold, *ca.* 3 ml./liter of a 0.5% aqueous solution being employed for this purpose. Used directly, or when added to the growth medium, it stained vegetative myxamoebae a very light blue, whereas aggregating myxamoebae and the cells of developing macrocysts assumed somewhat darker shades and appeared less granular.

Experimental Results

Origin and morphology of macrocysts

Macrocysts are flattened, irregularly circular to ellipsoidal multicellular structures, ranging from 25 to 50 μ in diameter. The myxamoebae which contribute to their formation appear normal for *Dictyostelium mucoroides* and *D. minutum* in every respect as they move and feed upon bacterial cells, re-dividing until the available food supply is exhausted. As this occurs, the myxamoebae begin to aggregate into pseudoplasmodia which, except for their generally limited dimensions, appear basically similar to other pseudoplasmodia that proceed to sorocarp formation (Fig. 4). However, instead of producing upright sorocarps, the myxamoebae comprising these aggregates remain in compact heaps and subsequently become surrounded by comparatively thick, cellulose walls. When only a few myxamoebae aggregate to form pseudoplasmodia, small macrocysts develop singly; when larger numbers of cells are involved, the macrocysts are somewhat larger and may occur in groups of varying size, ranging from small packets to

sheet-like ribbons, depending upon the number of myxamoebae massed together. In these larger aggregations, incipient macrocysts are delimited by the secretion of delicate cellulosic membranes around limited groups of cells more or less regularly spaced throughout the primary aggregate. This is followed by the subsequent deposition of thick, predominantly cellulosic walls, mostly circular to oval in pattern, that become the relatively rigid boundaries of the individual macrocysts. Commonly the secondary wall is laid down in general conformity with the primary membrane, but not infrequently two, three or even more macrocysts develop within an initial area of demarcation (Figs. 5–7).

Somewhat prior to the first evidence of secondary wall formation around the nascent macrocyst, cells in the center of the previously undifferentiated mass exhibit signs of modification and become surrounded individually by strongly refractive membranes. This process of cellular differentiation advances outward until all of the cells comprising the macrocyst are transformed into closely packed, seemingly firm-walled cells, termed *endocytes* (Figs. 8–9). The constitutive myxamoebae show no obvious orientation during the early stages of this process; but as differentiation proceeds, the peripheral and still amoeboid cells become conspicuously elongate as if appressed against the surface of the steadily enlarging body of endocytes (Fig. 6). Parallel with this progressive differentiation of the endocytes, but in a manner not yet understood, the whole body of functionally integrated cells succeeds in building around itself a tough and relatively heavy wall that is rich in cellulose. Significantly, the position of this wall is not determined by that of the thin primary membrane which initially delimited the bloc of myxamoebae that *might* collaborate in macrocyst formation; rather it is determined by the group(s) of cells which first differentiate as endocytes. This is clearly evident from the examination of primary aggregates of different dimensions. If the aggregate is small, the subsequently formed macrocyst wall will conform generally to that of the primary membrane. If the aggregate is relatively large, several centers of endocyte formation will arise simultaneously, and outward from these loci, cells will differentiate progressively to form separate and independent macrocysts, each with its own characteristic heavy wall but all contained within the primary membrane (Figs. 7–9, 25–27). The entire process of macrocyst formation is normally completed within 18 to 24 hours. Successive stages in macrocyst formation are illustrated in Figures 4 through 9.

The endocytes may be isodiametric or slightly elongate, ranging in size from about 3.6 to 4.8 μ in diameter. As observed within the macrocyst, and when first released by breaking the macrocyst wall, these cells normally appear polyhedral in outline, but soon become spheroidal or ellipsoidal when no longer compressed by adjacent endocytes (Fig. 9). For reasons still unknown they are appreciably smaller than the myxamoebae which enter the primary aggregate, the latter usually ranging between 6.0–8.0 μ in the unexpanded state. The smaller dimensions of the endocytes may result from a substantial water loss during their differentiation, or the contributing myxamoebae may possibly undergo division prior to the development of the refractive membranes which so strikingly distinguish them from other cells still amoeboid (Fig. 8).

Endocytes normally remain as distinct cellular entities within the macrocyst for a period of two or three weeks (Fig. 10), after which they commonly lose their identity and the protoplasmic content of the entire structure assumes a homo-

PLATE II. Origin and structure of macrocysts in *Dictyostelium mucoroides*, Strain S-28b.

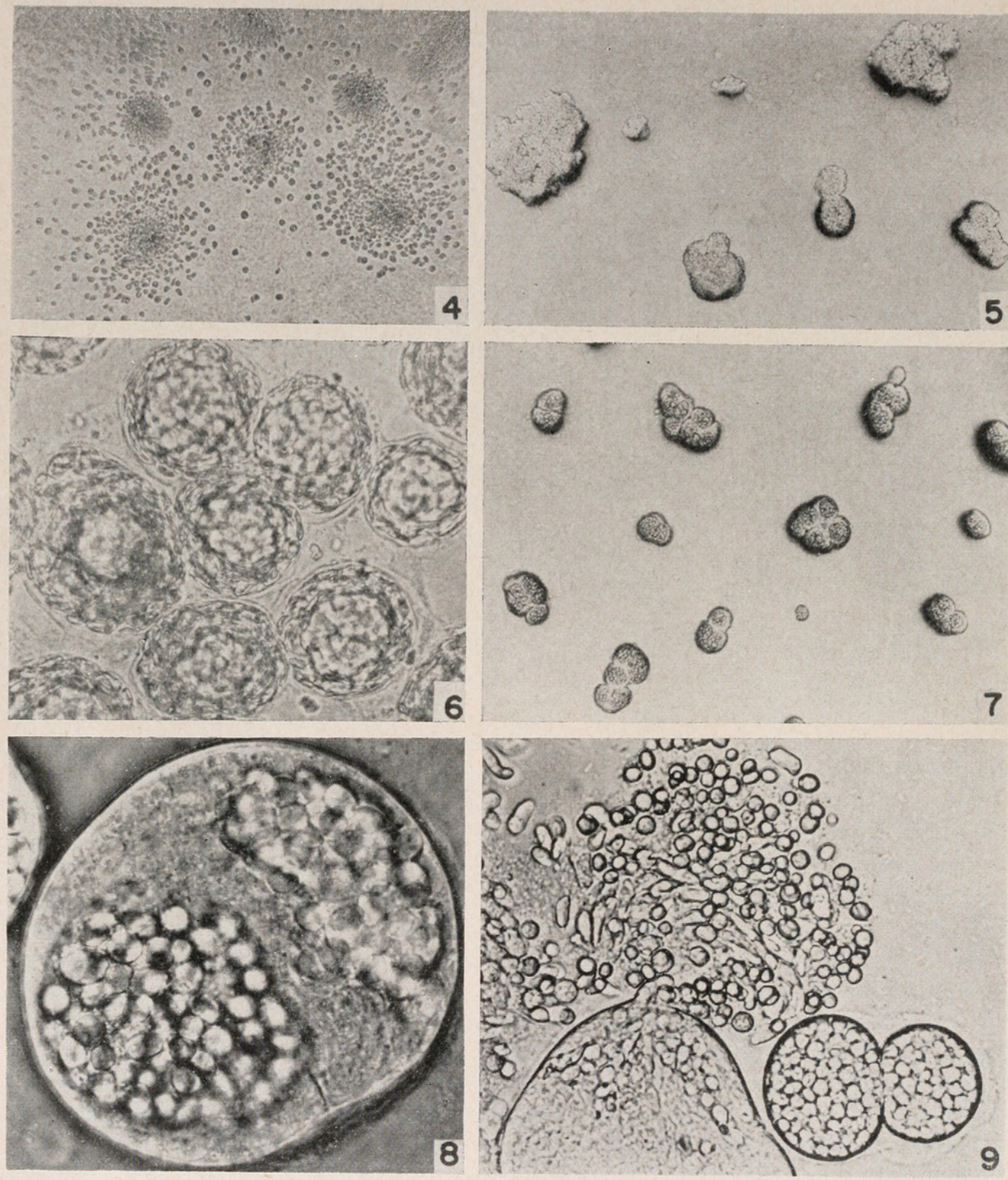


FIGURE 4. Small radiate pseudoplasmodia which lead to the formation of macrocysts. × 80.

FIGURE 5. Completed aggregations, consisting of irregular mounds of myxamoebae, prior to the formation of the primary membranes that delimit incipient macrocysts. × 80.

FIGURE 6. Enlarged view of a later stage in macrocyst development, showing incipient macrocysts and the orientation of their constituent myxamoebae. × 360.

FIGURE 7. More advanced stage showing clusters of differentiated endocytes centrally located in developing macrocysts. × 80.

FIGURE 8. Much enlarged view of two macrocysts in process of formation within a single

geneous appearance. With further aging, from four to six weeks, the apparently acellular content often shrinks to approximately 60–75% of its original volume, and in the form of a compacted, brownish mass occupies a central position within the partially empty macrocyst wall (Fig. 13). The surface of this central body appears slightly irregular and affords no clue to the presence of a continuous bounding membrane; rather it suggests a plasmolyzed and shrunken mass that has been subjected to uneven pressures and tensions during the process of contraction.

Many questions remain unanswered concerning the sequence of events leading to the advanced acellular structures just described. We have inadequate information concerning their true nature, and we have only incomplete knowledge of their significance in the life cycle of those slime molds where they occur. Nevertheless, a detailed study of macrocysts of different ages, and under many conditions, has revealed a considerable body of information concerning their development and behavior. If an endocyte-filled macrocyst is subjected to pressure in an aqueous mount, the heavy cellulose wall breaks, much as a hollow rubber ball, and the endocytes pour out, undergoing the limited changes in shape already noted but retaining their identity as relatively firm-walled cells. In contrast, if an older macrocyst from which the discrete endocytes have disappeared is similarly crushed, the enveloping wall breaks in a comparable manner, but the entire content flows out as a structureless fluid containing innumerable fine particles that immediately exhibit brownian movement as they enter a more aqueous environment.

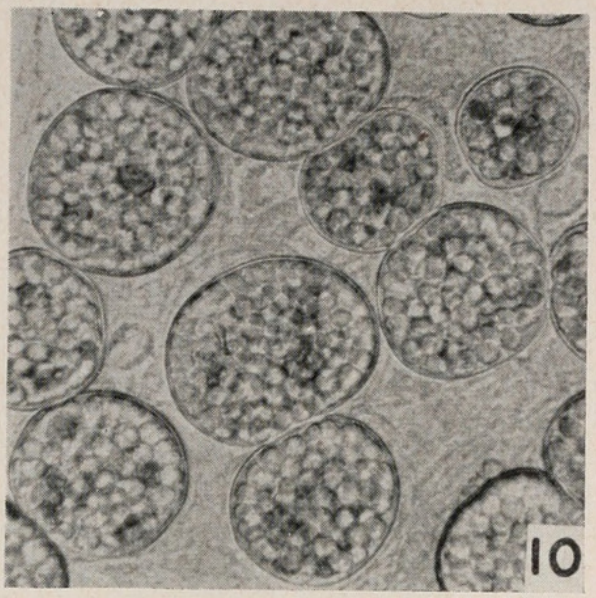
The explanation for endocyte disappearance in naturally aged macrocysts remains unknown, but a superficially similar state can be produced artificially with alkaline solutions. This was first observed when Schweitzer's reagent was applied to preparations of young macrocysts. Upon contact with the cuprammonium solution, the refractive walls of the endocytes disappeared, the seemingly merged content of the entire macrocyst swelled, and with the partial dissolution of the enveloping cellulose wall, the content emerged as a homogeneous and finely granular mass superficially resembling a large and completely undifferentiated protoplast (Fig. 17). A comparable disappearance of endocytes was subsequently observed in preparations following the application of NaOH (1.0 and 0.1 N), albeit the heavy cyst walls remained intact (Figs. 10–11). The application of HCl (1.0 N) to the same preparations prompted a most dramatic phenomenon, for upon contact with this reagent the endocytes reappeared in their original numbers and positions (Fig. 12). The same phenomenon of endocyte reversal accompanied the application of acid following treatment of young macrocysts with Schweitzer's reagent. If the exposed macrocyst was quite young the endocytes emerged individually as the cellulose wall dissolved, and these collected into a spreading amorphous mass within which cell boundaries remained faintly evident, as seen in Figure 16. If the cyst was older (but still packed with endocytes) the content emerged as an intact, seemingly homogeneous mass following similar treatment (Fig. 17). In each case a discrete cellular structure reappeared with the addition of acid (Fig. 18).

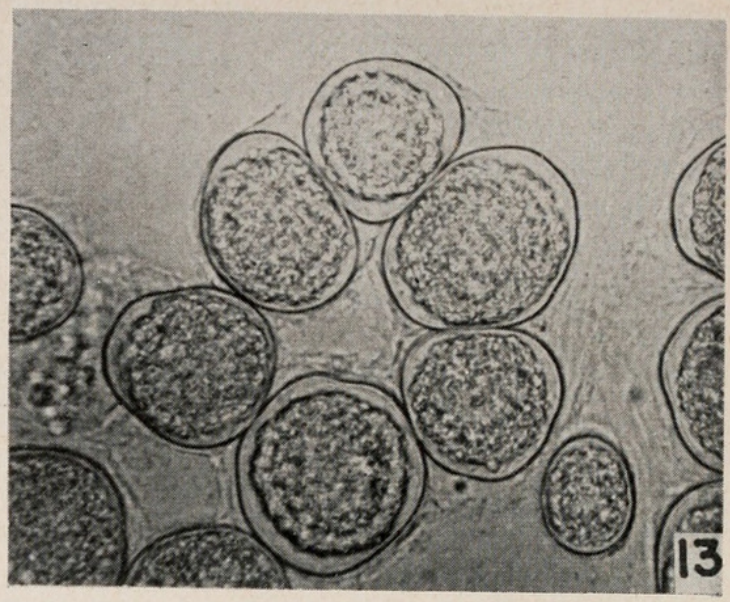
Macrocyst germination has been observed with the emergence of amoeboid cells from the ruptured cyst, and it was first thought that such induced disappearance

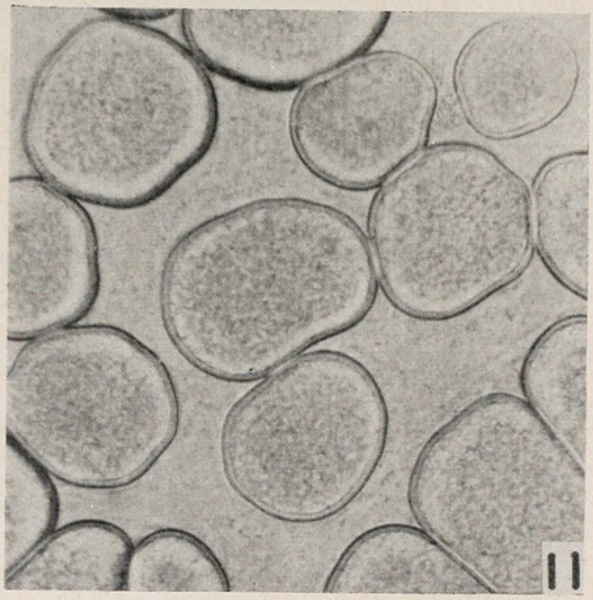
primary envelope, showing clusters of endocytes surrounded by undifferentiated myxamoebae. × 800.

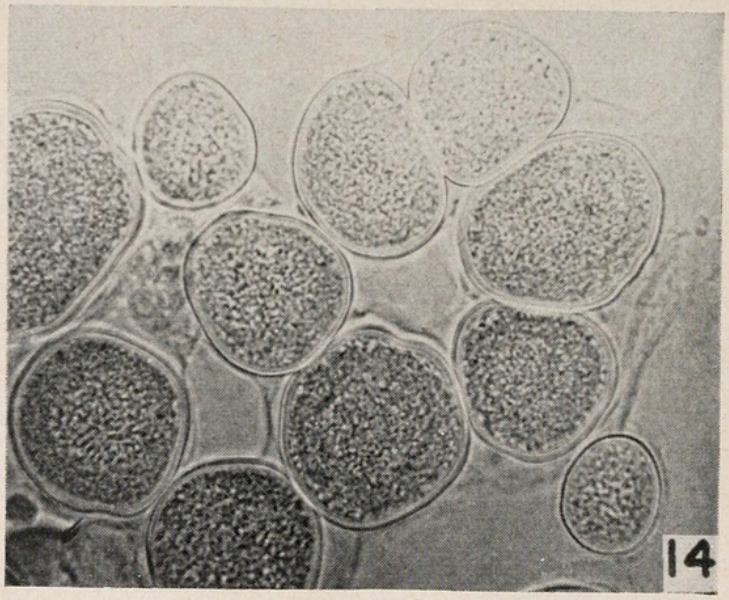
FIGURE 9. A body comparable to that shown in Figure 8, compressed to release endocytes and still undifferentiated cells. × 800.

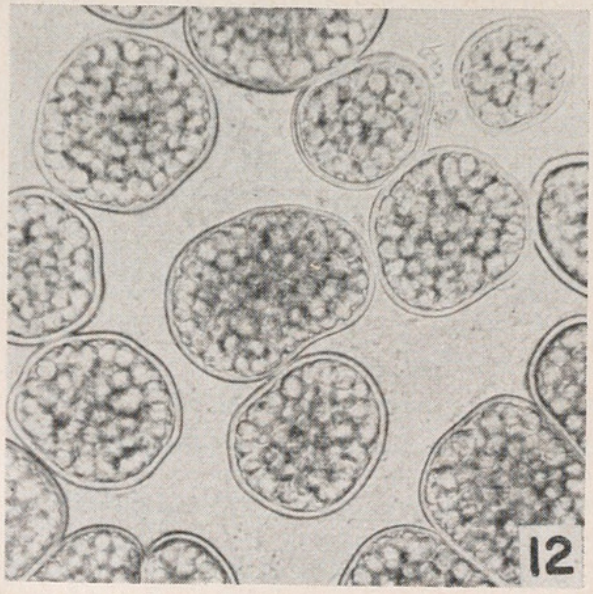
PLATE III. Behavior of young (endocyte-filled) and aged (homogeneous) macrocysts in *D. mucoroides*, Strain S-28b, in the presence of alkali and acid.

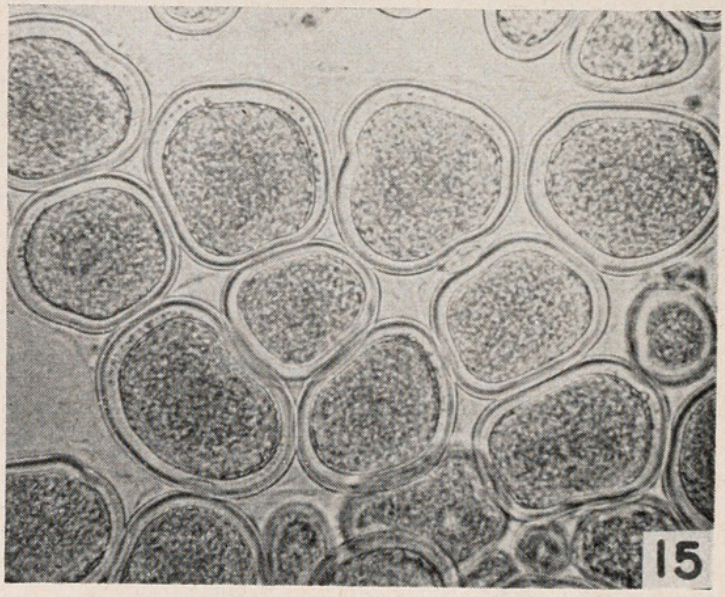












of endocytes might be analogous to the natural phenomena of macrocyst maturation and germination. However, further experiments failed to substantiate this view, for endocyte reversals did not occur at pH levels that permit growth and other vital activities of the slime mold. Furthermore, in the alternate presence of acid and alkaline solutions an initially endocyte-filled macrocyst could be interconverted repeatedly from an obviously cellular to a seemingly homogeneous state. Such reversal undoubtedly represents a physico-chemical reaction rather than a vital phenomenon, since macrocysts pre-killed with iodine-alcohol or Schaudinn's fixative react as do untreated structures.

The aforementioned tests in combination with certain others have provided substantial information regarding the wall structure of the macrocysts, and specimens treated alternately with alkali and acid and stained with chloroiodide of zinc, followed by Schweitzer's reagent, have proved particularly revealing. The "wall" of a mature, endocyte-filled macrocyst is seen to consist of three strikingly different parts: (1) an outer, loosely fitting primary covering of indeterminate form which characteristically surrounds one or more macrocysts, and may be continuous with, or adherent to, comparable envelopes of adjacent structures (Figs. 24–26); (2) a very much thicker secondary layer that is uniform in thickness, smooth in contour, and usually circular or ellipsoidal in outline (Fig. 26); and (3) an inner membrane formed by the endocytes prior to their disappearance as discrete cellular entities (Fig. 22). Both the primary covering and the secondary wall contain cellulose, apparently embedded within a matrix of more resistant material (Fig. 24–25). Both stain blue in chloroiodide of zinc and both are birefringent when viewed with polarized light, yet neither is completely dissolved by Schweitzer's reagent. However, each loses its birefringence upon the addition of the cuprammonium or an aqueous solution of 72% H_2SO_4. The primary membrane contains relatively little cellulose, embedded within a mucus-like material, and is reminiscent of the slime track and the slime sheath seen in *D. discoideum;* in contrast, cellulose constitutes the principal building substance of the thick secondary wall (Figs. 23 and 29), just as it does in the sorophore sheath of *Dictyostelium* (Raper and Fennell, 1952). The tertiary wall, if it may be so designated, contains no cellulose and represents a continuous film formed in a peripheral position by the mass of differentiating endocytes. This thin, innermost layer is non-rigid and contracts or expands with changes in the volume and character of the protoplasmic material that

PLATE III

FIGURE 10. Ten-day-old macrocysts produced on 0.1 L–P medium containing Nile blue sulfate, untreated. × 360.

FIGURE 11. The same macrocysts following exposure to 1.0 N NaOH; note that all evidence of the constituent endocytes has disappeared. × 360.

FIGURE 12. The same macrocysts following the application of 1.0 N HCl; note how the endocytes have reappeared. × 360.

FIGURE 13. Six-week-old macrocysts showing the typical contracted homogeneous content of such structures, untreated. × 360.

FIGURE 14. The same macrocysts following exposure to 1.0 N NaOH; note how the contents have swelled. × 360.

FIGURE 15. The same macrocysts, displaced in their relative positions, following the application of acid; note that the cyst contents have contracted somewhat, but that they show no evidence of a cellular structure. × 360.

PLATE IV. Behavior of young and aged macrocysts in Schweitzer's reagent.

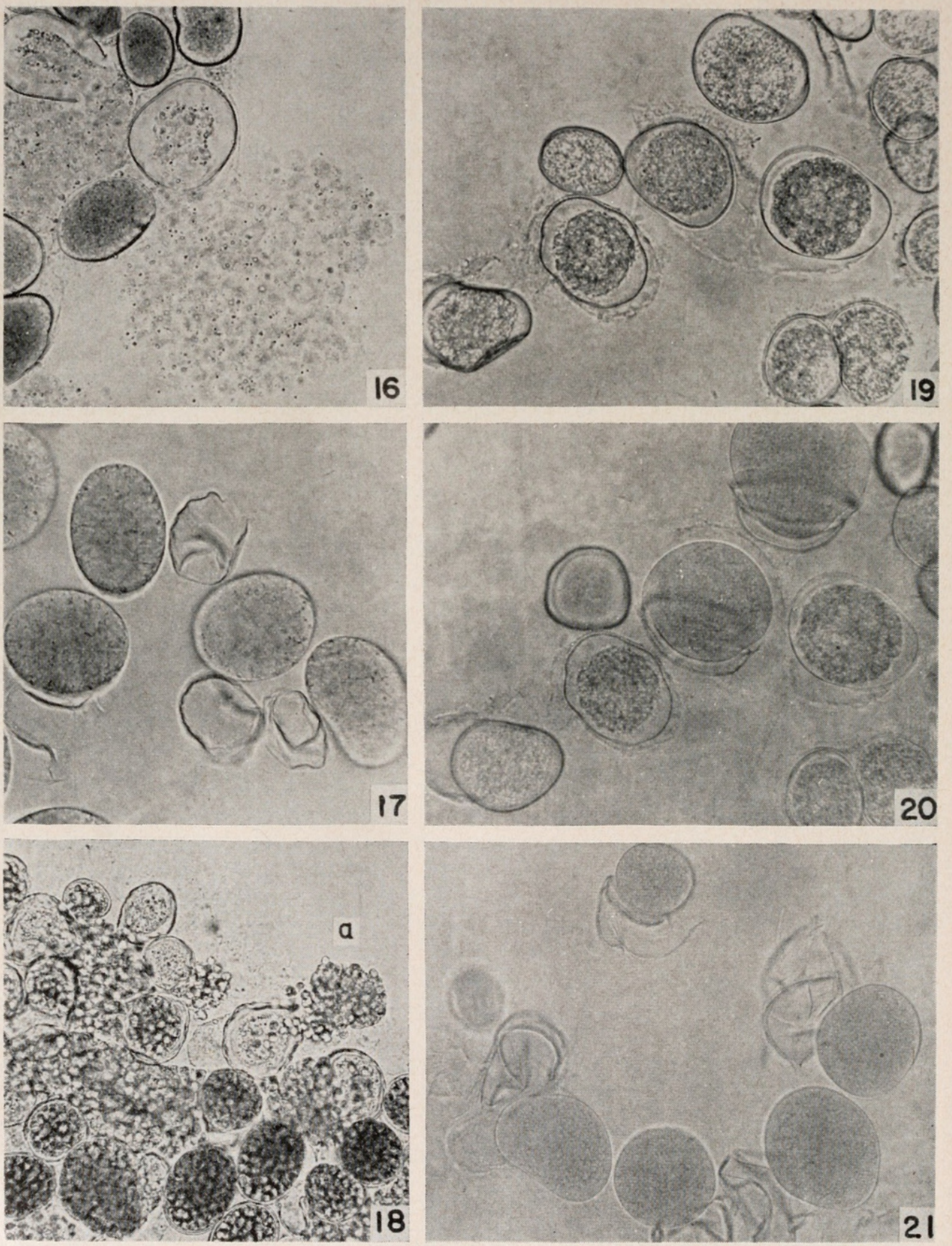


FIGURE 16. Young (endocyte-filled) macrocysts following exposure to Schweitzer's reagent, showing how the cuprammonium solution dissolves the cellulose walls of the macrocysts allowing the endocytes, faintly discernible, to escape. × 360.

it envelops, functioning as the tenacious covering that confines the merged cytoplasmic content of the aged macrocyst when this is released by dissolution of the heavy cellulose wall (Fig. 21). In contrast, when an aged but untreated cyst is broken by mechanical pressure this covering obviously ruptures with the cellulose wall which surrounds it (see above).

Similar preparations afforded equally interesting if more perplexing observations concerning the behavior of the contracted acellular protoplasmic masses present in the older macrocysts. When exposed to NaOH the shrunken content lost the brownish color characteristic of aged cysts and swelled to fill completely the heavy macrocyst wall. Upon the substitution of acid, the brownish pigmentation returned in part and the content again contracted, but not to its former dimensions (Figs. 13–15). Treated with Schweitzer's reagent the content swelled once more and erupted from the disintegrating thick cellulose wall as a single, seemingly homogeneous mass. Significantly, no return to a cellular structure has been observed in the content of any older cysts treated with cuprammonium or NaOH solutions upon the addition of acid (Fig. 15). Thus, there is evidence that the endocytes actually disappear, either by fusion or disintegration, at the time the aging macrocyst assumes a homogeneous appearance. But why does the emergent content remain intact following treatment with cuprammonium (Fig. 21) instead of flowing out freely as when the wall of the untreated homogeneous macrocyst is broken? Does the protoplasmic content represent, in fact, a plasmodium-like mass formed by the fusion of endocytes? Possibly so. Does the macrocyst wall actually consist of three layers, the innermost persisting about the freed protoplasmic body, not because of its inherently greater strength but because it is cuprammonium-resistant? Possibly this is true, for upon the application of slight pressure this bounding membrane breaks, permitting the fine granular content to escape whilst the membrane *per se* remains as a delicate, irregularly wrinkled and contracted envelope exhibiting no birefringence. Alternatively, and more plausibly, if aged macrocysts remain viable, as observations indicate, the thin hyaline envelope thus demonstrated may, in the living state, represent only the inconspicuous and functionally modified protoplasmic membrane of the contracted central body itself.

Two lines of evidence point to the presence of such a semipermeable membrane at progressive stages in macrocyst development. When an endocyte-filled macrocyst is exposed to a saturated solution of NaCl or sucrose, the content as a whole appears to become plasmolyzed and to withdraw from the surrounding thick cellulose wall as seen in Figure 22, plainly demonstrating the presence of a continuous

FIGURE 17. Older macrocysts (but still endocyte-filled) following exposure to this reagent; note how the nearly homogeneous cyst contents remain intact, and how the formerly heavy macrocyst walls are shrunken following the dissolution of their cellulose content. × 360.

FIGURE 18. Macrocysts of the same age as those shown in Figure 16, treated with Schweitzer's reagent and then exposed to 1.0 N HCl; note how the endocytes have reappeared, even in the freed contents of a macrocyst (a). × 250.

FIGURE 19. Two-month-old macrocysts, untreated. × 360.

FIGURE 20. The same macrocysts following treatment with Schweitzer's reagent; note how the outer cellulose wall is being dissolved and how the membrane-encased content is escaping intact. × 360.

FIGURE 21. The same preparation after an additional 10 minutes, showing the intact cyst contents completely free of the macrocyst walls; the latter are no longer birefringent when viewed with polarized light. × 360.

PLATE V. Macrocyst structure.

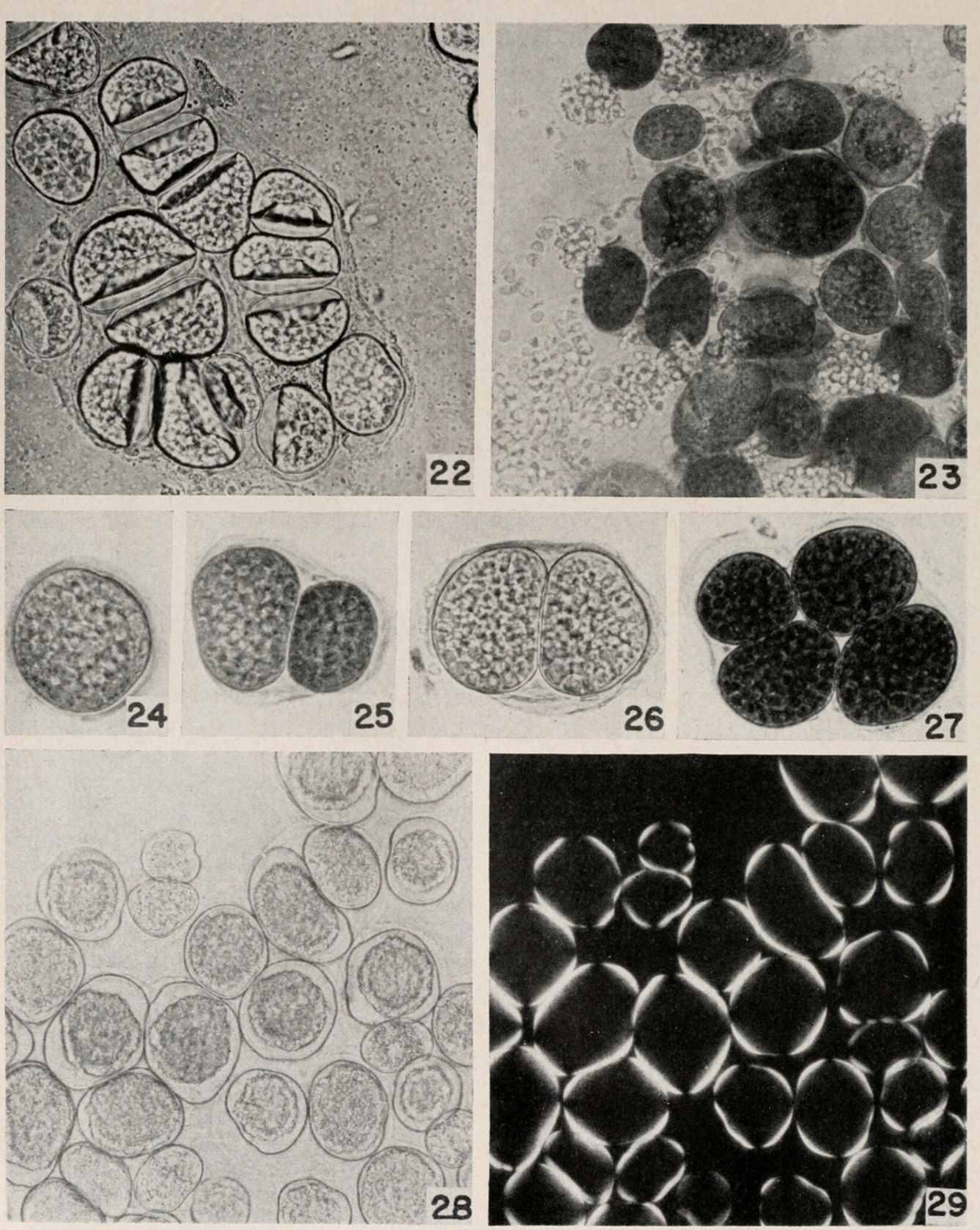


FIGURE 22. Young (endocyte-filled) macrocysts in the presence of a concentrated sucrose solution; note how a membrane, the "tertiary wall," surrounds the endocytes and in the process of plasmolysis pulls away from the rigid cellulose wall which is external to it. × 360.

FIGURE 23. Macrocysts of comparable age stained with chloroiodide of zinc and then subjected to pressure to release the endocytes. × 300.

and differentially permeable membrane external to the constituent endocytes but internal to the heavy cellulose wall. The same unitary pattern of plasmolysis is seen in macrocysts recently turned homogeneous, and in these there is no suggestion of persistent identity for the contributory myxamoebae or endocytes as one might expect if they remained as indistinguishable but nonetheless discrete cells. Aged macrocysts likewise provide contributory evidence. When placed in relatively large volumes of distilled water such structures show variable response depending upon the temperature of incubation. In preparations held at 10° C. evident swelling of the contracted homogeneous content is observed within 72 hours, and after 10 days many empty macrocyst cases, together with abundant free myxamoebae, may be observed (Figs. 41–42). At 15° C. little swelling occurs, even after 10 days, and only an occasional empty case may be seen. At 20 to 25° C. these responses are almost completely lacking (Fig. 40). This behavior is interpreted to indicate a selective permeability mediated by a low and favorable incubation temperature, and it is most unlikely that this could be attributable to the more conspicuous cellulose wall. The evidence would seem to point, indisputably, to the membrane that surrounds the shrunken cyst content.

The formation of macrocysts is observed not infrequently in *Dictyostelium minutum,* but their occurrence in strains diagnosed as *D. mucoroides* is relatively rare. In fact, not more than a half-dozen such isolates have been encountered among the hundreds of strains of the latter species that we have examined. In view of this, it is surprising and noteworthy that Brefeld (1869), in his description of *D. mucoroides,* reported objects believed to be similar to the macrocysts described above. His cultures were grown on microscope slides, and these structures developed in older preparations of that type. He described the structures as "dwarfed sporangia," since at times there was evidence of a rudimentary stalk in the "spore-forming plasm." He reported this type of sporangium to be enclosed by a comparatively thick membrane of cellulose, which upon examination was observed to be stratified and was stained violet with chloroiodide of zinc. This account agrees well with our observations of macrocysts submitted to various tests. He made no mention of the germination of "spores" from the "dwarfed sporangia." Rudimentary stalks such as Brefeld described and illustrated (Fig. 3) have not been observed during these investigations, and we believe that he may have observed immature macrocysts and interpreted clusters of differentiating endocytes as representing rudimentary stalks. In our experience, endocytes first appear in a localized central position within the developing macrocyst, but occasionally such cells do extend to the periphery along a particular radius before comparable differentiation occurs throughout the macrocyst. However, we must not overlook the possibility that, in his particular isolate cultivated under different conditions, he may have encountered stages truly transitional between macrocysts and well-formed sorocarps comprised of sorophores and sori. Certain evidence points to such a possibility. The basic similarity of the aggregative processes leading to sorocarp

FIGURES 24–27. Selected preparations stained with chloroiodide of zinc to show the loosely fitting primary membranes within which 1, 2 or 4 macrocysts have developed; the bodies shown in Figure 27 are older, hence the heavy macrocyst walls stain darkly. × 360.

FIGURE 28. Two-month-old macrocysts as viewed with normal light. × 275.

FIGURE 29. The same as seen under polarized light. × 275.

PLATE VI. Influence of substrates and incubation temperature upon macrocyst formation in *D. mucoroides,* Strain S–28b.

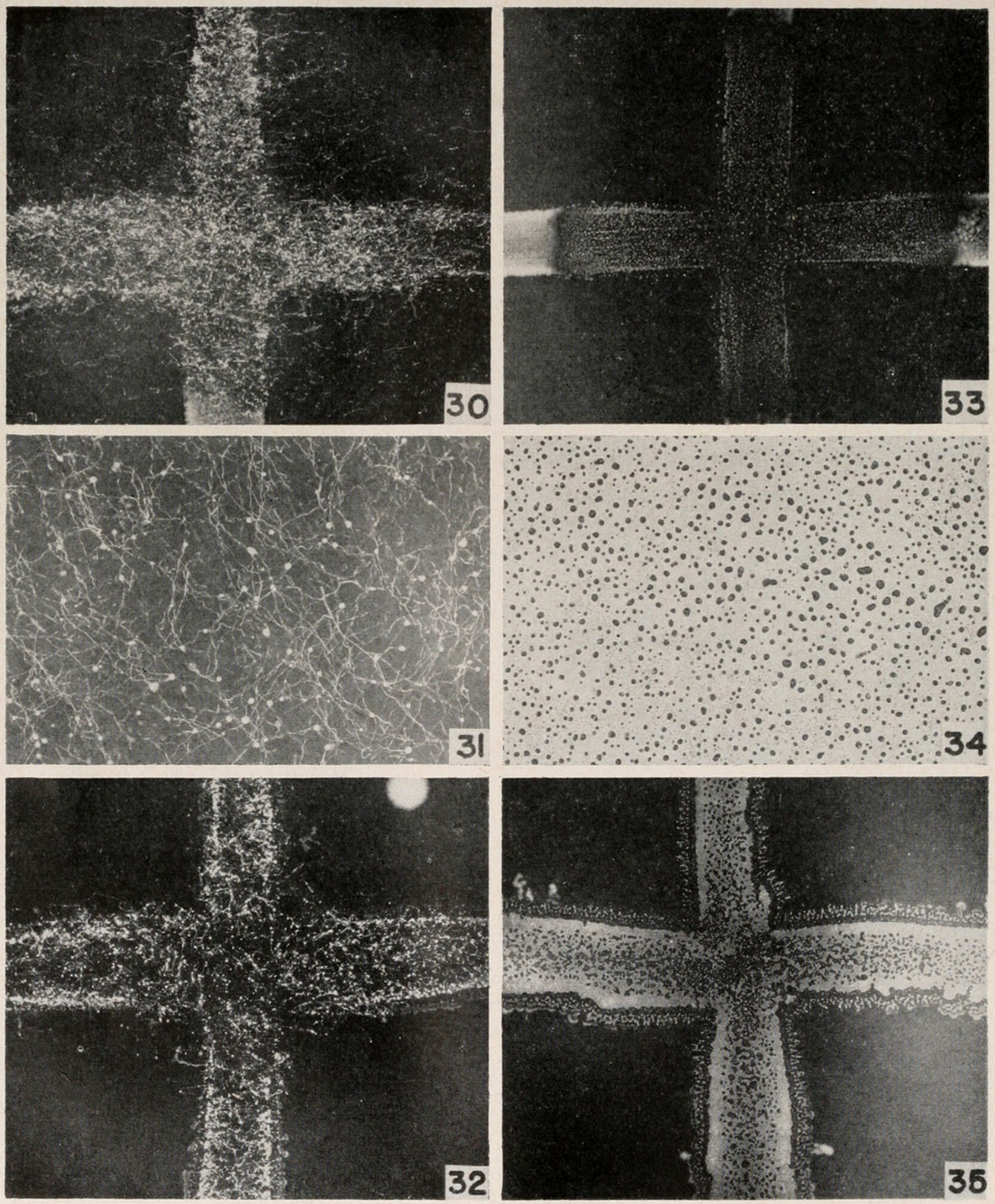


FIGURE 30. "Spore-forming clone" grown in association with *E. coli* on 0.1 L–P medium at 20° C. × 2.

FIGURE 31. Enlarged view of a portion of this culture showing abundant sorocarps and a complete absence of macrocysts. × 5.

FIGURE 32. The same culture as seen in Figure 30, but growing upon a medium containing 0.1% glucose-0.1% yeast extract, at 20° C. × 2.

formation, on the one hand, and to macrocysts on the other is indeed striking, and we have commonly observed in a single microscopic field separate pseudoplasmodia undergoing differentiation in these two directions simultaneously. Such a situation is illustrated in Figure 36. Unfortunately, we have no adequate explanation of the subtle differences that underlie this contrasting behavior, but it is easily conceivable that one might encounter individual cases where the shift from one to the other of these morphogenetic processes would be incomplete, as suggested by some of Brefeld's illustrations.

The formation of macrocysts may in effect represent an aberration of the normal fruiting process, or it may represent an alternative pattern of differentiation with implications of far-reaching significance that are yet unappreciated. The heavy cellulose wall which it develops bears in many ways a striking resemblance to the sorophore sheath, so essential to the construction of the normal sorocarp (Raper and Fennell, 1952), but with this important distinction: the wall of the macrocyst is secreted external to the whole mass of myxamoebae that contribute to the formation of this body, whereas the sorophore sheath is produced at a critical circular locus within the mass by a limited group of specialized cells which subsequently differentiate as the vacuolate cellular elements of the sorophore itself.

We have, at present, an incomplete picture of the morphology of the macrocyst. We have convincing evidence that they can germinate and re-initiate the life cycle of the slime mold. However, we do not know the fate of the cells which enter the macrocyst, and we do not know the origin of those which subsequently emerge. Until such information is at hand we cannot compare in any definitive sense the morphogenetic processes that underlie these contrasting developmental stages. A thorough cytological and histological study is clearly needed and will be undertaken at the earliest possible opportunity.

Factors influencing the formation of macrocysts

The obvious factor most directly affecting macrocyst formation in *Dictyostelium mucoroides* and *D. minutum* is the inherent genetic constitution of the particular isolate. Within our experience, a minority of *D. minutum* strains and only an occasional isolate of *D. mucoroides* have exhibited this capacity. However, once this ability has been demonstrated for a culture, it is sometimes possible to alter markedly the relative proportions of macrocysts and of normal sorocarps by changing the conditions under which the slime mold is cultivated. The responses of *D. mucoroides,* strain S–28b, have been studied in considerable detail and the observations subsequently recorded apply particularly to that strain. Whereas the various factors that influence the ratios of sorocarps to macrocysts are invariably interrelated, certain conditions which strongly affect these balances have been identified and investigated more or less independently.

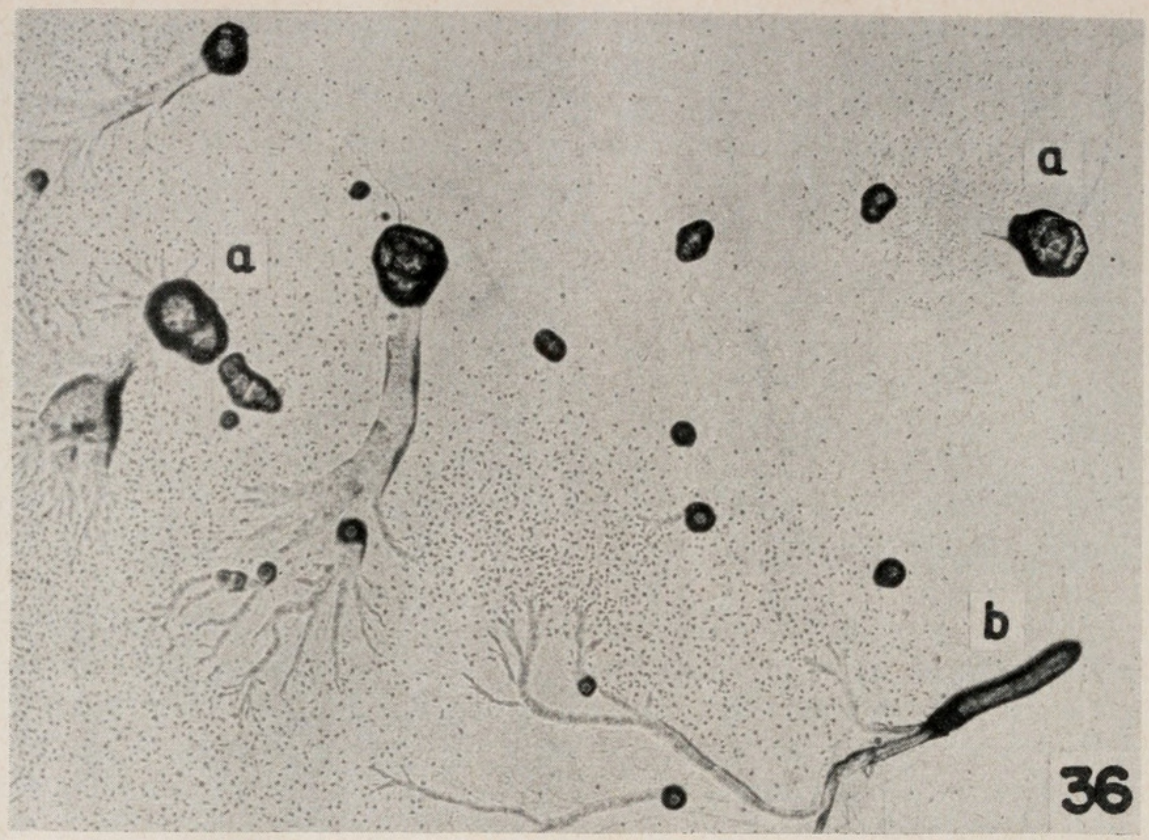
Temperature: Second only to genetic constitution is the influence of the incubation temperature. When cultivated at 24–25° C. in association with *Escherichia*

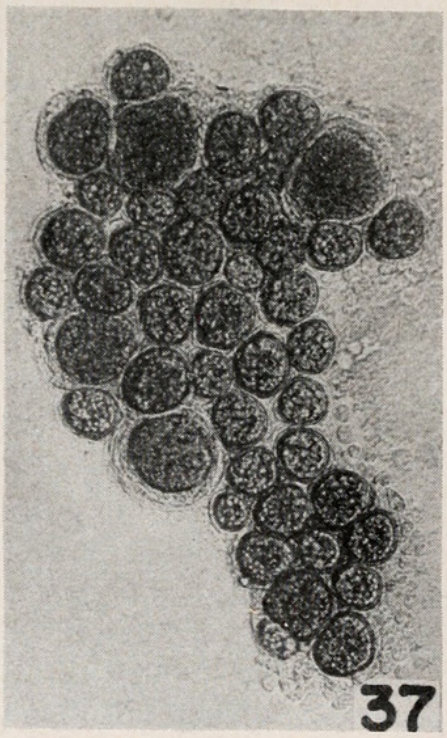
FIGURE 33. "Cyst-forming clone" growing in association with *E. coli* on 0.1 L–P medium at 20° C. × 2.

FIGURE 34. Enlarged view of a portion of this culture showing abundant macrocysts and a complete absence of sorocarps. × 7.5.

FIGURE 35. The same culture as seen in Figure 33, but growing upon a medium containing 0.1% glucose-0.1% yeast extract at 20° C. × 2.

PLATE VII. Macrocyst formation in *Dictyostelium mucoroides*, Strain WS–47.





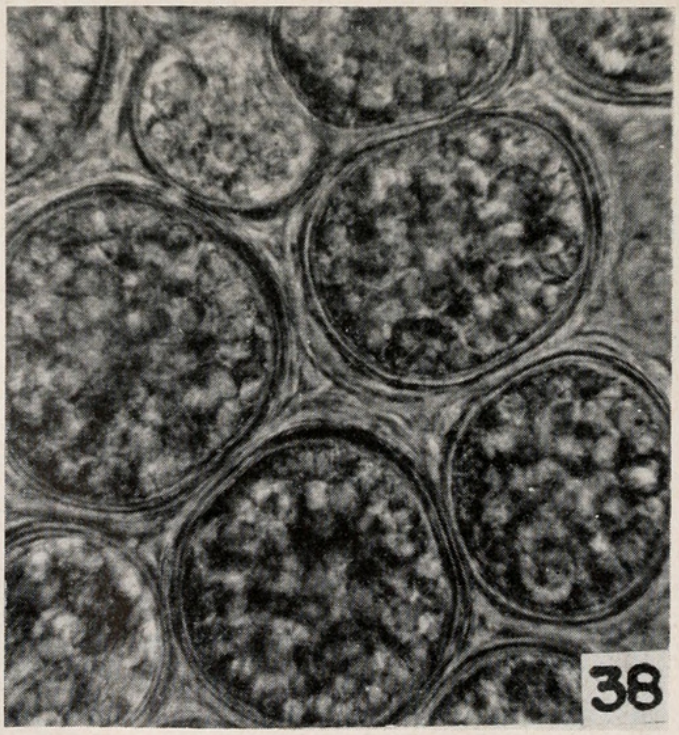


FIGURE 36. Radiate pseudoplasmodia leading to macrocyst formation (a) and to sorocarp formation (b) within the same microscopic field, as seen with low magnification. × 24.

FIGURE 37. Irregular clump of macrocysts developing from a single pseudoplasmodium such as that shown in the preceding figure. × 125.

FIGURE 38. Detail of some of the macrocysts seen in Figure 37. × 600.

PLATE VIII. Macrocyst germination in *Dictyostelium mucoroides*, Strain S–28b.

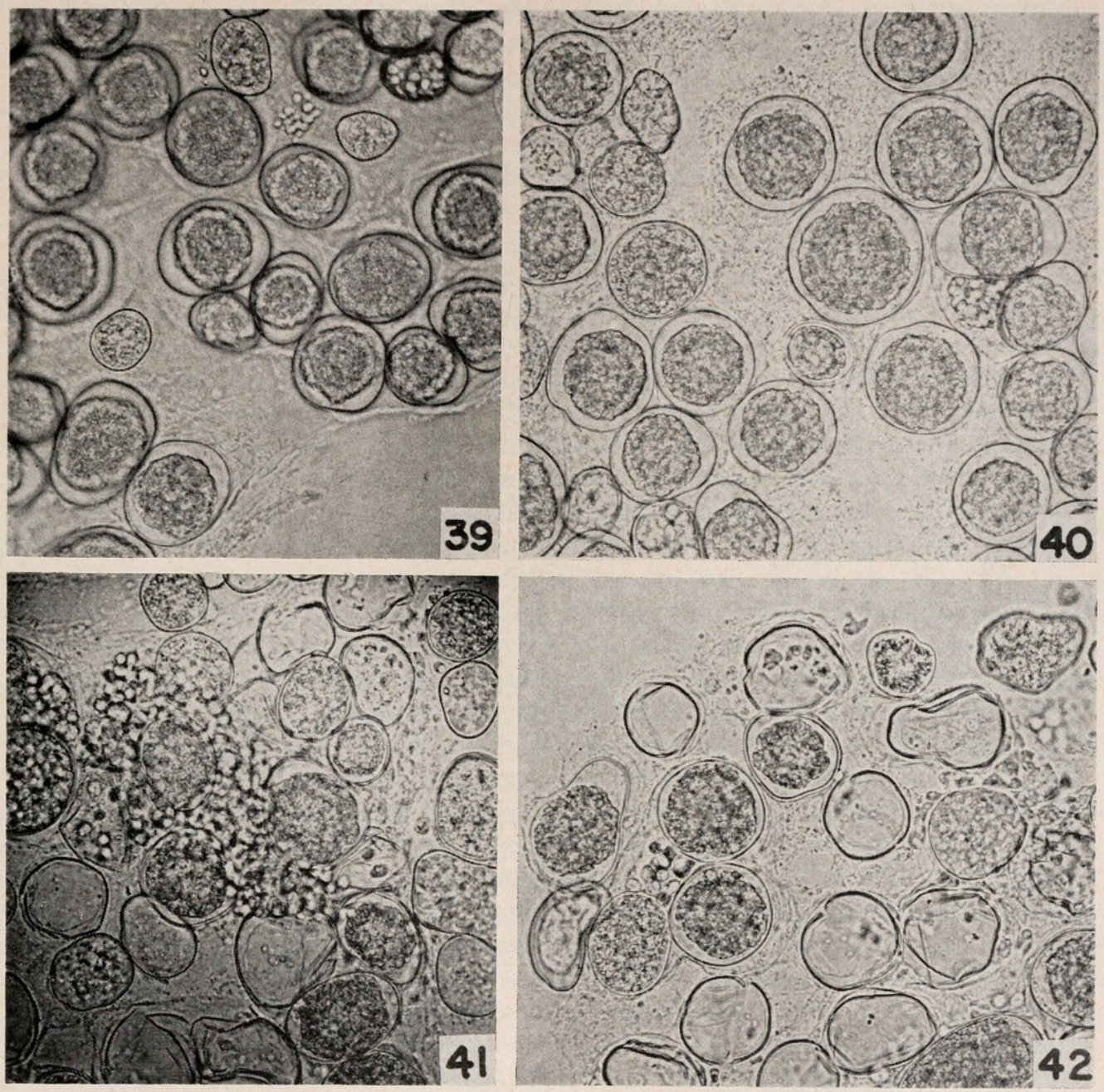


FIGURE 39. Two-month-old macrocysts incubated continuously at 20° C. and removed from an agar plate immediately before being photographed; note the single macrocyst at upper right which still contains endocytes. × 300.

FIGURE 40. Macrocysts similar to the preceding, but removed from agar and incubated in distilled water at 20° C. for 10 days prior to photography; note that little evident change has taken place. × 300.

FIGURE 41. Macrocysts like the preceding, but incubated for 10 days at 10° C. just prior to being photographed; note the empty cyst cases and the mass of free myxamoebae which have escaped from these. × 300.

FIGURE 42. Photomicrograph showing a second field from the same preparation shown in Figure 41; note the broken walls of the empty cyst cases, also that a few myxamoebae still remain within the germinating cyst at top center. × 300.

coli on 0.1 L–P, hay-infusion or thin hay-infusion agars, the stock strain of S–28b regularly produces both sorocarps and macrocysts in abundance, although individual cultures and even different areas within the same Petri dish commonly exhibit conspicuously disproportionate ratios of these contrasting structures. If the temperature is raised as little as two or three degrees the ratio of macrocysts to sorocarps is increased substantially; conversely, if it is lowered to 20° C. or less this ratio is strongly depressed.

Clonal substrains of S–28b have been isolated which exhibit temperature sensitivity even more dramatically. These were obtained by heating spore suspensions in standard salt solution (Bonner, 1947) for varying lengths of time and then, following appropriate dilution, plating the spores in association with *Escherichia coli* on 0.1 L–P agar. Plaques, evidenced by clearance of the bacteria, developed after four days in plates incubated at 20° C., presumably from single spores. Some of these subsequently developed only macrocysts, others produced only sorocarps. By re-isolation from such contrasting areas, a "cyst-forming clone" and a "spore-forming clone," with strikingly different temperature responses, were isolated (Figs. 30–35). Upon continued recultivation on 0.1 L–P agar, the former characteristically produced only macrocysts at 20° C. but developed abundant sorocarps and scattered macrocysts at 15° C., whereas the latter typically produced only sorocarps at 20° C. but formed both sorocarps and macrocysts at 25° C. Cultures initiated with spores from the cyst-forming clone grown at 15° C. developed macrocysts when recultivated at 20° C., demonstrating a marked degree of genetic specificity in the myxamoebae and spores of the clone. Thus the pattern of cellular differentiation exhibited by different clones of S–28b results from inherited characteristics that are temperature-dependent for their expression.

Culture media: The production of macrocysts is strongly influenced by the substrates upon which the slime mold is cultivated, and these structures are regularly formed in greater abundance upon 0.1 L–P agar than upon media based upon hay-infusion. Their development is even further accentuated if yeast extract is substituted for peptone, this being used either as the sole nutrient or in combination with lactose or glucose. For example, upon 0.1% lactose-yeast extract or 0.1% glucose-yeast extract agars abundant macrocysts are formed by the cyst-forming clone incubated at 15° C. and by the spore-forming clone at 20° C., in each case at temperatures where few or no macrocysts normally develop on 0.1 L–P agar. An intimate association obviously exists between substrate composition and incubation temperatures as these factors affect macrocyst formation, but just how they condition the cultural environment and how they affect the fructifying myxamoebae, influencing them to produce either macrocysts or sorocarps, has not been determined (Figs. 30–35).

Bacterial associate: Dictyostelium mucoroides, S–28b, can be cultivated successfully with a variety of bacterial associates on the nutrient-poor media employed in this investigation. Gram-negative bacteria support better growth of the slime mold than do Gram-positive types, and of the former *Escherichia coli* was the most favorable species investigated. *Aerobacter aerogenes, Pseudomonas fluorescens* and *Serratia marcescens* yielded satisfactory but less luxuriant slime mold growth. The formation of macrocysts and sorocarps followed, in general, the patterns observed with *E. coli.* The myxamoebae of strain S–28b digest or destroy the red pigment (prodigiosin) of *S. marcescens,* hence yield uncolored sorocarps and

macrocysts. In marked contrast, the cells of strain NC–12, like those of *D. discoideum,* retain the pigment (Raper, 1937), hence produce sorocarps and macrocysts that are pink in color. *Bacillus subtilis,* a Gram-positive bacterium, supported fair growth of the slime mold and the formation of abundant macrocysts on 0.1% lactose-yeast extract agar at 20° C. *Sarcina lutea,* a second Gram-positive species, permitted only limited growth of myxamoebae and few macrocysts were produced on any substrate. An experiment conducted to determine what effect the age of the nutritive *E. coli* might have on growth and macrocyst formation in *D. mucoroides* failed to reveal any significant differences.

Hydrogen-ion concentration: A heavy suspension of *E. coli* cells previously grown in 0.1 L–P broth and concentrated by centrifugation was cross-streaked on 0.1 L–P agar plates to investigate the effect of pH on slime mold growth and macrocyst formation. The underlying agar substrate was adjusted over a range of pH from 3.3 to 9.2 using the buffers previously cited. In these experiments growth of myxamoebae was obtained between pH 4.5 and 8.0. Slight evidence of aggregation was observed at pH 4.5 but no well-defined pseudoplasmodia developed, and of course no sorocarps or macrocysts were produced. A heavy development of sorocarps and/or macrocysts was obtained between pH 5.5 and 7.0, depending upon the incubation temperature. No growth occurred above pH 8.0 where K_3BO_3 was used as buffer. Questioning whether it was the high pH or the borate buffer which inhibited growth, we made additional tests using KH_2PO_4 as buffer with the addition of NaOH to yield pH levels from 7.5 to 9.0. Growth of the stock culture occurred to pH 9.0, but few and abnormal sorocarps and no macrocysts were formed. Even in the cyst-forming clone incubated at 20° C. where one would normally expect only macrocysts, many sorocarps and very few macrocysts developed on the more alkaline substrates. The pH of the medium did not change appreciably during the period of slime mold growth.

Ammonia concentration: Cohen (1953) reported that ammonia suppressed normal morphogenetic development in the Acrasieae, either inhibiting growth completely, or causing various abnormalities in sorocarp formation. His procedures were carefully followed to ascertain whether different concentrations of ammonia would enhance macrocyst formation. It was thought that if these structures represented aberrant sorocarps, their production might be enhanced under conditions where sorocarp formation was inhibited. However, macrocysts were not formed in any of the ammonia concentrations employed, possibly indicating a suppressive effect comparable to that reported for sorocarps by Cohen.

Relative humidity: The effect of per cent relative humidity on slime mold growth and the development of macrocysts was tested by the procedures previously indicated. Large depression and ordinary flat slides with one ml. of 0.1 L–P agar were employed, rather than Petri dishes, to insure rapid establishment of an equilibrium between the moisture of the agar and the atmosphere in the desiccators. Olive (1902) attributed the encystment of myxamoebae (microcysts) to adverse conditions such as the drying of culture substrates. If macrocyst formation should represent a stage comparable to the encystment of individual myxamoebae, we would have anticipated more macrocysts when the per cent relative humidity was lowered, causing a drying of the substrate. Such did not occur in our tests.

Macrocyst formation in other strains: Attempts were made to enhance macrocyst formation in other strains of *D. mucoroides* (WS–47 and NC–12) and in *D.*

minutum (WS–56–2 and Purdue 8a). Cultural conditions were varied much as previously outlined for *D. mucoroides,* S–28b. However, in these additional slime molds neither the type of culture medium nor the concentration of its ingredients seemed to markedly affect the relative number of macrocysts produced. For example, the proportion of macrocysts to sorocarps remained relatively constant upon media containing yeast extract *vs.* peptone and upon nutrient-rich *vs.* less concentrated media, although total growth of the bacteria and of the slime mold varied substantially with such changes.

A variety of culture media were buffered and adjusted between pH 6.0 and 8.5. All supported growth of the slime mold, and in no case was there an exceptional increase in macrocyst production.

Temperature relationships were examined carefully. *Dictyostelium mucoroides,* WS–47 (Figs. 36–38), produced more macrocysts at 20° and 25° C. than at 15° C., but this response was not so striking as in strain S–28b. A few macrocysts developed in strain NC–12 at 20° C., but at 25° C. only irregular growth of the slime mold occurred, indicating too high an incubation temperature. In *D. minutum,* Purdue 8a, approximately equal macrocyst formation was observed at 20°, 25° and 28° C. In contrast, WS–56–2 formed no macrocysts at 20° C., only a very few at 25° C., and grew very poorly at 28° C.

Germination of macrocysts

The possible role of the macrocysts in the life cycle of *Dictyostelium* engaged our attention from the outset, since it did not seem reasonable that such structures produced in great abundance and under seemingly optimal conditions would represent a terminal and functionless kind of differentiation. For this reason much thought and effort have been given to their germination. In this, as in other phases of the investigation, studies have been centered upon *D. mucoroides,* S–28b, and our researches have been greatly facilitated by the cyst-forming clones. By capitalizing upon their unique temperature responses it has been possible to produce at will large populations of macrocysts under cultural conditions where no spores were formed. At the same time any vegetative myxamoebae which might have adhered to the macrocysts were readily killed by heating at 42° C. for 10 minutes. No growth of the slime mold occurred when the macrocysts were heated at a temperature high enough to kill the mature spores, and such would have been present in any macrocyst preparations taken from the parent culture. The comparative temperature tolerances of the myxamoebae and spores of *D. mucoroides,* S–28b, are shown in Tables I and II, respectively.

Proceeding on the assumption that the macrocysts might represent a resistant stage in the cycle of *Dictyostelium,* they were subjected to a variety of cultural environments and treatments. Plates of 0.1 L–P agar containing abundant macrocysts were alternately frozen at − 10° C. and thawed at + 25° C. in an attempt to instigate germination, but consistent results were not obtained. Growth from macrocysts treated in this way was twice observed after ten days' incubation. Crump (1950) had reported that raising the temperature favored the germination of encysted free-living amoebae, but no germination ensued after heating macrocysts in a water bath for five minutes at 55° C.

At the suggestion of the late Dr. Charles Thom, an attempt was made to stimu-

late macrocyst germination by spreading a heavy inoculum of pre-grown cysts and *E. coli* on sterilized soil in Petri dishes and incubating the plates at 10°, 15° and 20° C. No evidence of germination was obtained in 28 days, although we had demonstrated previously that growth of the slime mold could take place under these natural conditions.

Brefeld (1869) stated that germination of *Dictyostelium* spores took place only in a nitrogenous medium and suggested fresh horse dung decoction and uric acid media as substrates. Both were investigated. No evidence of macrocyst germination was observed on fresh horse dung agar plates, but growth was obtained, in association with *E. coli,* on 0.05% uric acid agar after eight days incubation at 15° C.

TABLE I

*Temperature tolerance of myxamoebae of Dictyostelium mucoroides, S-28b, suspended in distilled water**

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test		
		1	2	3
0	42	++++	++++	++++
5		0	+	+
10		0	0	0
15		0	0	0

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
+ = Limited growth.
0 = No growth.

* Similar results were obtained when myxamoebae were suspended in standard salt solution.

Macrocysts were dismembered in a McShan-Erway tissue homogenizer in the hope that the endocytes thus freed would re-initiate growth under suitable cultural conditions. Such homogenates were mixed with *E. coli* and the resulting suspensions spread on 0.1 L–P agar plates. No growth of the slime mold ensued, but the possibility of serious injury to the endocytes could not be discounted.

Despite the negative results obtained, the seeming logic of this approach led us yet again to attempt the dissolution of the macrocyst wall by other means as a possible aid to germination and regrowth. The procedure employed was probably ineffective *per se,* but in performing the experiment the "treated" macrocysts were incubated at a variety of temperatures which provided a clue to cultural conditions where germination not infrequently occurred. A cellulase preparation, contributed by Dr. Emory G. Simmons, was investigated as a means of digesting the heavy enveloping macrocyst wall. The enzyme was employed as a 1% solution in *M*/20 citrate solution at pH 5.0. Macrocysts were harvested from cultures where no spores had formed, suspended in the cellulase-citrate solution, and heat-treated to kill any adherent myxamoebae. Germination occurred in varying amounts in the cellulase-treated macrocysts and also in controls similarly heat-treated in standard salt solution. The earliest evidence of germination was observed after 6 days'

incubation, and new plaques continued to develop for as long as 22 to 40 days at varied temperatures. The percentage germination was approximately the same for macrocysts heat-treated in the cellulase-citrate and in the standard salt solutions. The earliest evidence and the greatest amount of germination were observed among the macrocysts incubated at 15° C., with decreasing amounts to 25° C. Actual

TABLE II

Temperature tolerance of spores of Dictyostelium mucoroides, S-28b, suspended in standard salt solution (Test 1) and in distilled water (Tests 2, 3, and 4)

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test			
		1	2	3	4
0	42	++++			
90		++++			
0	50	++++	++++		
65		+	+		
0	55	++++	++++		
5		++	+++		
10		0	+		
15		+	+		
20		0	+		
25		0	0		
30		0	+		
35		0	0		
0	60	++++	++++	++++	++++
5		+++	++	+	++
10		+	++	+	0
15		0	++	0	0
20		0	+	0	+
25		0	+	0	0
30		0	0	0	0
0	65	++++	++++	++++	
5		+	+	0	
10		0	0	0	
15		+	0	0	
20		0	0	0	

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
\+ = Limited growth.
0 = No growth.

germination of a macrocyst, or of the endocytes contained within it, was not then observed, but in some instances one or more empty macrocyst cases were evident where a plaque of growth occurred.

It was now hoped that even though the percentage viability was apparently low, a few macrocysts might be seen to germinate if these were carefully isolated and observed periodically over a period of several days. Single macrocysts of dif-

ferent ages (3, 15 and 35 days) were selected, heat-treated to kill any vegetative myxamoebae, and placed individually in marked squares on 0.1 L–P agar plates smeared with *E. coli*. Evidence of macrocyst germination was noticed after incubation at 15° C. for five days.

A series of experiments was undertaken to determine whether macrocysts of a particular age would germinate more readily than cysts of other ages; and since presumptive germination had seemed to vary at different incubation temperatures, special consideration was given to this matter. To obtain macrocysts of specific ages, a heavy suspension of myxamoebae of the cyst-forming clone and *E. coli*

TABLE III

Percentage germination among macrocysts of Dictyostelium mucoroides (No. S-28b) of different ages, and the number of days before such germination was observed at different incubation temperatures

Age of macrocysts, days	Temperature of incubation									
	5°		10°		15°		20°		25°	
	Days	%	Days	%	Days	%	Days	%	Days	%
5 (1)*	35	0	35	0	35	0	35	0	35	0
(2)*	44	0	18	0.6	30	0.2	30	0.07	38	0
10 (1)	35	3.4	20	3.1	22	7.2	28	4.5	42	0
(2)	30	+**	14	+	14	3.0	25	0.7	30	0
15 (1)	38	1.6	18	2.9	12	1.2	18	5.5	38	0
(2)	43	0	20	+	20	+	20	+	37	0
20 (1)	25	0.5	18	1.8	12	1.0	12	1.0	32	0.2
(2)	38	0	23	1.0	15	0.9	15	1.0	29	0
25	38	+	15	4.0	15	+	18	1.0	30	0
35	28	+	20	+	8	0.2	8	0.1	20	0.07

* Indicates separate experiments.

** (+) Indicates germination of macrocysts, but percentage of total population could not be calculated.

was spread over the surface of 0.1 L–P plates and incubated at 20° C. for 5, 10, 15, 20, 25 and 35 days. These macrocysts were then harvested, heat-treated to eliminate all vegetative myxamoebae, spread on fresh agar plates with *E. coli,* and incubated at 5°, 10°, 15°, 20° and 25° C. for 5 to 6 weeks. The results of these experiments are summarized in Table III.

Substantial growth of the slime mold was obtained in certain of the above tests, particularly in plates inoculated with 10- and 15-day-old macrocysts incubated at intermediate temperatures, the highest percentage (7.2%) being observed in 10-day cysts incubated at 15° C. The prevalence of empty macrocyst cases in the developing plaques, the prior heat-treatment of the cysts to kill adherent myxamoebae, the carefully prepared and examined source plates from which the macrocysts were

taken for these experiments, and the observed presence of empty macrocyst cases at central locations within many plaques, all convinced us that the observed growth must have developed from germinated macrocysts.

Nevertheless, we had not actually observed this phenomenon, and even in the most consistent macrocyst-forming culture it is possible that a minute sorocarp could go unobserved and that an occasional spore, which would not be killed by heating to 42° C., might be carried over with the implanted macrocyst. An experiment was carried out to determine whether an occasional spore, if present, might have served as the initiator of the plaques of amoeboid growth in the macrocyst germination plates. It was known that growth from spores would eventually occur at all of the incubation temperatures employed (5° to 25° C.); therefore, the times required for plaques to develop from individual spores under cultural conditions duplicating the above were determined. At 20° C. plaques were evident within 4 days, at 15° and 25° C. within 6 days, at 10° C. within 11 days, while at 5° C. growth was not evident until 25 days. Since plaque formation is usually optimal at 15° C. on the macrocyst germination plates, and does not become evident until after 8 to 11 days, these results provided additional evidence that the observed growth resulted from macrocysts and not from occasional contaminating spores.

Having determined the optimum cyst age and the incubation temperature that are favorable for macrocyst germination, 10-day macrocysts in association with *E. coli* were implanted on freshly poured plates and on sterile Maximov slides containing 1.0 ml. of 0.1 L–P agar to observe the germination of the macrocysts directly. Realization of this objective proved unexpectedly time-consuming, but it was accomplished. Germination in this instance, as in the majority of cases observed up to this time, was from a macrocyst filled with endocytes at the time of implantation in the test culture. Pre-germination changes were not observed but it is assumed that the heavy macrocyst wall was ruptured either by swelling of its content and/or by enzymatic dissolution (see below).

Re-examination of Table III reveals that maximum germination occurred among the 10-day-old macrocysts; *i.e.*, structures which were packed with endocytes at the beginning of the tests. More significantly, appreciable germination was recorded for some of the older macrocysts, notably the 25-day cysts incubated at 10° C. This result is especially interesting since cysts of this age would have already lost their discrete endocytes, in the great majority of cases, and would have assumed the homogenous appearance that characterizes aged cysts. Thus presumptive evidence was obtained that macrocysts of the latter type are capable of germination—presumptive because even in preparations taken from cultures after several weeks occasional macrocysts are seen in which the endocytes remain distinct, and the recorded germination could have resulted from such non-homogeneous structures. The improbability of this explanation was subsequently demonstrated. Blocks of 0.1 L–P agar bearing abundant homogeneous macrocysts aged 6 and 8 weeks were placed in sterile Petri dishes, flooded with sterile distilled water, and incubated at 10°, 15°, 20°, and 25° C. Within only 10 days approximately half of the cysts of both ages incubated at 10° C., and only at this temperature, had germinated among populations where prior examination had established that only occasional cysts (2–4%) were still in the endocyte stage at the beginning of the experiment (Figs. 39–42). An understanding of the intracystic events which transpire during the progression from endocyte differentiation to their subsequent disappearance,

and from this through the stage of seeming homogeneity and protoplasmic contraction to the eventual swelling of this mass and the reappearance of amoeboid cells at the time of germination, must await careful cytological investigation. For the present we can only record that in the presence of a proper aqueous environment and at a favorable temperature the previously shrunken "protoplast" (long believed doubtfully viable) swells and gives rise to myxamoebae which escape during cyst germination. Additionally, there is evidence that these myxamoebae, or the parent coenocyte (?), produce cellulolytic enzymes which facilitate rupture of the heavy cyst wall, for viewed with the polarizing microscope the empty cyst cases contain conspicuously less cellulose than do the walls of macrocysts still ungerminated.

Formation and germination of microcysts

Many members of the Acrasieae are characterized by a second, simpler type of encystment stage where individual vegetative myxamoebae round up and become encased by relatively thin protective membranes (Fig. 1). The walls of these resting cells, or microcysts, like those of the macrocysts and the more resistant spores, are predominantly cellulosic in composition. As reported by Olive (1902), there is ample evidence that these form in response to sub-optimal growth conditions. It is probable that the myxamoebae of any member of the Acrasieae may enter such a stage temporarily, but they are most commonly encountered, and in greatest numbers, in isolates of *Dictyostelium minutum, D. polycephalum, Polysphondylium pallidum,* and *Acytostelium leptosomum.*

Microcysts of several species of the Acrasieae were examined to determine their method of germination. Microcysts were placed in hanging-drop slides in thin-hay broth with killed cells of *E. coli* and incubated at 25° C. Within two days many of the microcysts had germinated, as evidenced by the number of free, feeding myxamoebae and by the empty microcyst cases from which these had emerged. Previous workers (Olive, 1902) had not reported true excystment of the microcysts, but had intimated that the myxamoebae absorbed the protective covering, or wall, during germination. The emptied cases are extremely delicate and hyaline, and some reveal a fairly obvious opening at one side through which the myxamoeba escaped. They do not germinate by the emergence of the protoplast through a pore or exit tube, neither do they appear to split as do the spores of most species; rather, germination appears to take place by the dissolution of a fractional portion of the microcyst wall. The cyst cases stain violet-blue with chloroiodide of zinc and show a weak birefringence, indicating that they contain some cellulosic material.

In *Dictyostelium mucoroides* microcysts are about twice the dimensions of the endocytes that comprise the macrocysts, ranging from about 5.0 to 7.5 μ in diameter and being generally spheroidal.

Discussion

Intriguing questions are posed by the macrocysts of *Dictyostelium* with regard to their morphogenesis and their probable primary function in the life-cycle of these slime molds. Do they represent a normal but generally unrevealed stage in the

development history of the Acrasieae, *i.e.*, could they be demonstrated in all members of the group if we but knew the conditions required to evoke them? Do they provide a resting stage whereby these micro-organisms survive otherwise impossible environmental conditions? Do they perhaps constitute some unanticipated manifestation of a sexual stage? Or do they represent, as their superficial appearance might suggest, groups of myxamoebae so thwarted in their normal morphogenesis that they become "captives" doomed to a type of terminal differentiation approximating that of sterile stalk cells? Does the identity of the contributory myxamoebae remain unchanged during the formation of the endocytes, and do the latter in some altered form persist to once again emerge during germination as myxamoebae capable of perpetuating the species? For certain of these questions we have succeeded in providing partial answers.

Brefeld illustrated some "dwarfed sporangia" that contained differentiated cells which he interpreted as representing elements of abortive stalks, and early in this investigation we questioned whether the endocytes might not in fact reflect a type of cellular differentiation of this type. More careful examination has established beyond question that such is not the case despite certain superficial similarities in appearance. The walls of the endocytes contain no demonstrable cellulose and the content of such cells is actually condensed, whereas the walls of true stalk cells contain cellulose and the cell content is strongly vacuolate, occupying a peripheral position within the semi-rigid cell. Upon treatment with alkali (*e.g.*, 1.0 N NaOH) the walls of stalk cells do not disappear as do those of the endocytes.

Much evidence supports the belief that macrocysts arise through an orderly and natural morphogenetic process, and hence in no wise represent aberrant fruiting structures. For those strains which produce them, they would appear to be no less normal than the sorocarps which regularly develop under similar or, in some instances, altered conditions. A measure of homology is suggested by the basically similar aggregative process which precedes the formation of both types of structure. The pseudoplasmodia leading to macrocyst formation are generally diminutive, but this condition is not a necessary precedent to their formation. Additionally, the myxamoebae entering a pseudoplasmodium destined to form macrocysts rarely show the marked elongation characteristic of cells entering larger aggregations, but this weak cellular response is believed to indicate degree rather than difference, *i.e.*, to reflect a feeble aggregative stimulus incident to, or responsible for, the small pseudoplasmodium.

A point of similarity should be noted between the morphogenetic processes leading to the formation of gregarious sorocarps in certain species (*e.g.*, *D. minutum* and *D. lacteum*) and to clustered macrocysts in *D. mucoroides,* for in both situations the magnitude of the initial pseudoplasmodium often exceeds the number of myxamoebae that can effectively collaborate in producing a single sorocarp or macrocyst. In the former instance, secondary centers appear soon after the overall pattern of the wheel-like aggregate becomes evident, and from each of these a separate sorocarp subsequently develops; in the latter case, multiple loci of endocyte formation similarly appear within the initial aggregate, and from each of these later develops a discrete and typical macrocyst.

Substantial differences characterize subsequent steps in the two morphogenetic processes, and there is little if any evidence to suggest that the macrocysts represent modified or abortive sorocarps. The latter can be produced in any known species

by a variety of devices (*e.g.,* unfavorable pH, increased temperature, etc.), but in no observed instance have such abnormal fruits presented a pattern which is remotely suggestive of macrocyts. Rather, they assume the form of *Guttulina*-like fructifications wherein the myxamoebae produce irregular mounds and undergo incomplete differentiation, but they never form a common protective wall about the mass of cells so assembled.

The heavy cellulosic wall of the macrocyst bears a structural likeness to the sorophore sheath of the normal sorocarp, but, as noted earlier, the relative positions at which these are deposited by the constitutive cells are quite different. Furthermore, the formation of the sorophore sheath is antecedent to cellular differentiation in sorocarp building, whereas it lags behind this phenomenon in macrocyst construction. There is yet another difference which may prove highly significant. Bonner *et al.* (1956) demonstrated that the sorophore sheath is secreted by an ever-changing epithelium-like layer of myxamoebae that are oriented perpendicular to the surface of the wall being deposited, whilst in the macrocysts the last remaining amoeboid cells, hence those adjacent to the developing wall, are oriented in quite the opposite direction. Only in those cultures where seemingly identical and intermixed pseudoplasmodia give rise either to sorocarps or to macrocysts, as in strain WS–47, do we find evidence that the two morphogenetic pathways may be closely allied, and in these we have at present no concept of what major or minor organizational differences may underlie such divergence. Judging from Brefeld's illustrations (1869), it is possible that he may have seen so-called "dwarfed sporangia" that were transitional between sorocarps and macrocysts, but no structures of this type have been observed in our cultures. Finally, we would reiterate that the body of evidence presently available points to macrocyst production as representing an alternative but thoroughly normal morphogenetic pathway that is an inherited character possessed by occasional strains of *D. mucoroides* and by many isolates of *D. minutum.* The isolation of contrasting "cyst-forming" and "spore-forming" clones in strain S–28b strengthens this belief, as does also our inability thus far to induce macrocyst formation in any culture of *D. mucoroides* which did not naturally exhibit this capacity at the time of its isolation.

In contrast to this situation, the capacity to produce microcysts seems to be generally present among the Acrasieae, and it is suspected that every isolate may, under certain variable environmental conditions, exhibit such a resting stage. It should be recognized, however, that this phenomenon is probably totally unrelated to macrocyst formation. Microcysts represent the responses of single myxamoebae to effect a transitory resting stage in the vegetative phase of these slime molds and is perhaps strictly comparable to the encystment of certain small, free-living amoebae. Their natural function is indisputably one of enabling the species to survive otherwise unfavorable environments. The macrocysts, on the other hand, arise through multicellular integration and differentiation and represent the product of a specific morphogenetic process, just as do the sorocarps. This function is still incompletely known.

We have obtained convincing evidence that macrocysts germinate under certain circumstances, emitting amoeboid cells which then re-initiate vegetative growth. But we cannot say with confidence that the macrocysts represent a vital resting stage, as their appearance might suggest. Heat tolerance tests indicate that they can withstand appreciably higher temperatures than vegetative myxamoebae, but

they are in turn less resistant than true spores. While the matter has not been explored under conditions that exist in nature, it is possible that they might be produced under circumstances which would preclude the formation of sorocarps and spores, *e.g.*, in strains such as S–28b at elevated temperatures. The general application of this premise is doubtful, however, for many macrocyst-producing strains fail to show a comparable response. Based upon laboratory tests, we could not, at present, conclude that they possess singular survival value.

Possibly they are endowed with other unique properties, a suggestion presently based less upon fact than fancy. We find it difficult to dismiss lightly a structure of multicellular origin which appears to be so highly organized as the older macrocyst. We cannot say with absolute certainty that its content represents a single homogeneous multinucleate protoplast, but such tests as we have applied would seem to support this notion. If the endocytes do actually lose their identity, as appears to be the case, the acellular content of the aged macrocyst would represent a coenocyte, or to use a term more commonly associated with slime molds, perhaps a plasmodium, albeit one that is enclosed within a heavy cellulose wall. Such a plasmodium would of course be quite unlike that which Brefeld (1869) once thought to be present, or that which Skupienski (1920) envisioned as an accompaniment to reported sexuality in *Dictyostelium*. Needless to say, it would represent quite a different structure from the large vegetative body that occupies so conspicuous a place in the life-cycle of the Myogastrales. Clearly, the two could not be regarded as homologous. The same may be said of the plasmodial stages of the Plasmodiophorales, for although their dimensions would be more nearly comparable, these also are never characterized by heavy cellulose walls. Furthermore, no one has yet reported a swimming stage, either gametic or vegetative, for any member of the Acrasieae and such are generally precedent to the formation of plasmodia in each of the other orders.

Here the matter must rest for the present, and definitive information regarding the true nature and ultimate significance of the macrocysts must await further research.

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Summary

1. Two encystment stages of cellular slime molds belonging to the genus *Dictyostelium* are described:

The first of these, termed *microcysts,* are unicellular and represent a transient resting stage in the vegetative phase of these simple slime molds. If returned to a favorable environment, microcysts germinated by excystment to re-initiate vegetative growth.

The second encystment stage, termed *macrocysts,* are multicellular and arise through a morphogenetic process possibly alternative to normal sorocarp formation. Myxamoebae aggregate to form typical but generally diminutive pseudoplasmodia which, instead of forming normal sorocarps, subdivide into rounded cell masses that become encased in relatively heavy cellulose walls. Concurrent with this de-

velopment, the myxamoebae that comprise the nascent macrocyst undergo limited differentiation and appear as polyhedral cells with highly refractive membranes. After a period of 10 to 14 days these so-called *endocytes* generally disappear whereupon the content of the macrocyst assumes an acellular, homogeneous appearance. With further aging the protoplasmic content shrinks away from the heavy cellulose wall and in this contracted stage retains its viability for protracted periods. Under favorable conditions of temperature and substrates, macrocysts of different ages germinate to release amoeboid cells which re-initiate the vegetative stage. The sequence of cytological changes underlying this behavior has not been elucidated, and this propagative function may or may not represent the full measure of their significance in the life-cycle of those slime molds which produce them.

2. The ability to produce microcysts is apparently inherent in all members of the Acrasieae, including the genus *Dictyostelium*. In contrast, the capacity to produce macrocysts is more restricted, having been observed only in occasional isolates of *D. mucoroides* and in many strains of *D. minutum*. Various environmental factors influence their production, and from one strain of *D. mucoroides* temperature-dependent "cyst-forming" and "spore-forming" clones have been isolated.

LITERATURE CITED

Bonner, J. T., 1944. A descriptive study of the development of the slime mold *Dictyostelium discoideum*. *Amer. J. Bot.*, **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.*, **106**: 1–26.

Bonner, J. T., 1952. The pattern of differentiation in amoeboid slime molds. *Amer. Nat.*, **86**: 79–89.

Bonner, J. T., A. Duncan Chiquoine and Marjorie Quick Kolderie, 1955. A histochemical study of differentiation in the cellular slime molds. *J. Exp. Zool.*, **130**: 133–158.

Brefeld, O., 1869. *Dictyostelium mucoroides*. Ein neuer Organismus aus der Verwandschaft der Myxomyceten. *Abhandl. Senckenbergisch. Naturf. Ges. Frankfort, a/m,* **7**: 85–107.

Clark, W. M., 1928. The determination of hydrogen ions. Third Edition. The Williams and Wilkins Co., Baltimore.

Cohen, Arthur L., 1953. The effect of ammonia on morphogenesis in the Acrasieae. *Proc. Nat. Acad. Sci.*, **39**: 68–74.

Cormier, Joan L., and Kenneth B. Raper, 1955. The macrocysts of *Dictyostelium*. *Bact. Proc.* p. 36 (abst.).

Crump, L. M., 1950. The influence of bacterial environment on the excystment of amoebae from soil. *J. Gen. Microbiol.*, **4**: 16–21.

Hodgman, C. D., ed., 1951. Handbook of chemistry and physics. Thirty-third edition. Chemical Rubber Publishing Co., Cleveland, Ohio.

Olive, E. W., 1901. A preliminary enumeration of the Sorophoreae. *Proc. Amer. Acad. Arts Sci.*, **37**: 333–344.

Olive, E. W., 1902. Monograph of the Acrasieae. *Proc. Boston Soc. Nat. Hist.*, **30**: 451–513.

Raper, Kenneth B., 1935. *Dictyostelium discoideum,* a new species of slime mold from decaying forest leaves. *J. Agric. Res.*, **50**: 135–147.

Raper, Kenneth B., 1937. Growth and development of *Dictyostelium discoideum* with different bacterial associates. *J. Agric. Res.*, **55**: 289–316.

Raper, Kenneth B., 1940a. Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.*, **56**: 241–282.

Raper, Kenneth B., 1940b. The communal nature of the fruiting process in the Acrasieae. *Amer. J. Bot.*, **27**: 436–448.

RAPER, KENNETH B., 1941. *Dictyostelium minutum,* a second new species of slime mold from decaying forest leaves. *Mycologia,* 33: 633–649.

RAPER, KENNETH B., 1951. Isolation, cultivation, and conservation of simple slime molds. *Quart. Rev. Biol.,* 26: 169–190.

RAPER, KENNETH B., 1956a. Factors affecting growth and differentiation in simple slime molds. *Mycologia,* 48: 169–205.

RAPER, KENNETH B., 1956b. *Dictyostelium polycephalum* n. sp., a new cellular slime mould with coremiform fructifications. *J. Gen. Microbiol.,* 14: 716–732.

RAPER, KENNETH B., AND DOROTHY I. FENNELL, 1952. Stalk formation in *Dictyostelium. Bull. Torrey Bot. Club,* 79: 25–51.

SKUPIENSKI, F. X., 1920. Recherches sur le cycle évolutif des certains myxomycetes. Paris. (Published by the author.)

STEVENS, W. C., 1916. Plant anatomy. Third Edition. P. Blakiston's Son & Co., Philadelphia.

WILSON, R. E., 1921. Humidity control by means of sulphuric acid solutions, with critical compilation of vapor pressure data. *J. Ind. and Eng. Chem.,* 13: 326–331.