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Studies on the reactivating sclerotium of Physarella oblong morgan with special reference to the nucleus

William Thomas Allman

STUDIES ON THE REACTIVATING SCLEROTIUM OF *PHYSARELLA OBLONGA* MORGAN WITH SPECIAL REFERENCE TO THE NUCLEUS

by

William Thomas Allman Jr., B. S.
Richmond College
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INTRODUCTION

Introduction

The Myxomycetes, because of their naked, mobile mass of protoplasm have served biologists and chemists as material for the study of the characteristics of protoplasm. The typical life history of such an organism may be summarized as follows: The vegetative phase in the life history consists of a naked mass of protoplasm, to which Cienkowski (9) applied the name "plasmodium". This plasmodium creeps by reversible streaming over and throughout the substratum, deriving its food from various constituents of decaying vegetable matter which serve as its normal substrate. Upon reaching a state of maturity and under certain environmental conditions, the plasmodium is converted into fruiting bodies of various types such as sporangia, aethalia and plasmodiocarps. Within these fruiting bodies, typically uninucleate spores are formed. These spores are freed by a breaking down of the fructification wall and are disseminated by wind, rain, insects and other vectors.

Under favourable conditions of sufficient warmth and ample moisture the spore germinates as described

by Gilbert (13), Smart (35, 35A) and others, to form uniflagellated swarm cells. These swarm cells swim through the water for a time, during which they may feed by engulfing bacteria, spores and other particulate foods. Eventually two compatible swarm cells, upon coming in contact with one another, fuse, withdraw their flagella and form an amoeboid zygote. Jahn (18) was the first to suggest sexual fusion of these swarmers, followed by such investigators as Skupienski (34), Wilson and Cadman (40), Cayley (8), Howard (15), Abe (1), Smart (36), and others. The zygote thus formed, feeds, increases in size through assimilation and becomes multi-nucleated by repeated division of the zygotic nucleus, and gives rise to the plasmodium.

When certain conditions unfavourable for further activity overtake the plasmodium the protoplasm withdraws from its expanded strands and veins, loses water and forms a protective dormant stage to which De Bary (2, 3, 4) applied the term "sclerotium".

It is generally agreed that the sclerotium of Myxomycetes is formed from the plasmodium when the

humidity of the organism's environment is decreased to such an extent that irreparable damage to the plasmodium would result if certain protective measures were not taken. Numerous multinucleated cellular structures, spherical to polyhedral, probably approaching the minimal tetrakaidecahedron (6), are bound together. These cellular structures, called spherules by Brandza (5), constitute the sclerotium.

It has been shown by several students, i.e., Schinz (29), Léveillé (32), A. Lister (21), and others, that sclerotia may be retained in dry condition for several months to several years and upon returning them to favourable conditions of ample moisture and warmth they will give rise to active plasmodia. Thus the sclerotium, its structure and the conditions relative to its formation and reactivation, is worthy of careful study since the sclerotium provides a convenient means of maintaining experimental plasmodia in an inactive state.

The factors involved in inducing the sclerotization of plasmodia and the reactivation of sclerotia to active plasmodia have been studied by Smart (36) and the literature relative to such factors has been

carefully reviewed by him.

Smart's review of the literature and the results of his experiments reveal the fact that there is general agreement among the students of the Myxomycetes relative to conditions for sclerotization of plasmodia and for the reactivation of these sclerotia. On the other hand, a review of the literature reveals the fact that no such general agreement exists relative to the structure of the sclerotium and the behaviour of the nuclei during the time of reactivation.

De Bary (2) described the sclerotium as being made up of small protoplasts contained within a more or less definite membrane and taking on a shape which might be spherical, ovoid or polygonal, but he did not refer to the presence or condition of nuclei in the sclerotium. Van Tieghem (39) on the other hand regarded the sclerotium as a multicellular cyst and believed that there were as many spherules in the sclerotium as there were nuclei in the plasmodium from which the sclerotium had been derived.

Strasburger (38) after reviewing the literature

relative to the sclerotium came to the conclusion that the spherule membrane surrounding each protoplast may or may not be present depending upon the circumstances involved in the formation of the sclerotium.

A. Lister (21, 23) working with Badhamia utricularis Berk., found that each protoplast contained from ten to twenty nuclei, was surrounded by a membraneous wall and each cyst assumed an irregular shape and eventually dried to a horny mass.

M.C.A. Jorgensen (20) demonstrated by photographs the reactivation of a sclerotium to form a macroscopic plasmodium and the formation of the sclerotium from a plasmodium. He describes some details of the cyst, how the protoplasts abandon the protective membrane of the cyst and how the crystallization of calcium on the surface of the sclerotium of calcareous myxomycetes is effected.

Brandza (5) described the spherules of Badhamia macrocarpa Rost., Fuligo septica Gmel. var candida R. E. Fries F., Badhamia capsulifera Berk., Physarum pulcherrimum Berk. and Rav., P. contextum Pers., P. carneum List. and Sturg., P. psittacinum Dittm.,

Cienkowskia reticulata Rost., Craterium minutum Fries, Leocarpus fragilis Rost., Didymium difforme Duby., and Didymium complanatum Rost.

In all descriptions, with the exception of Didymium difforme Duby., Brandza figured individual spherules. In this species he demonstrated by drawings that the spherules which have concentric membranes and give the characteristic cellulose reaction with chloro-iodide of zinc, are widely separated from each other by delicate filamentous trabeculae. These trabeculae were not identified as to detailed structure nor as to function. He showed by figures that spherules of Fuligo septica Gmel., Badhamia capsulifera Berk., B. macrocarpa Rost., Physarum pulcherrimum Berk. and Rav., P. contextum Pers., Leocarpus fragilis Rost., Didymium complanatum Rost., and D. difforme Duby., may contain nuclei which stained intensely with hematoxylin and also contained "amoebulae". The outer diameter of these amoebulae, Brandza found to be two or three times that of the nuclei. He described the center of the amoebule as being occupied by a body which apparently stains with approximately the

same intensity as do the nuclei, and are identified by him as such. According to Brandza there are included occasionally as many as two "petite noyaux" per amoebule.

Brandza found that spherulization or sclerotization may be complete or partial. In order to completely sclerotize, the sclerotium must be derived from a young plasmodium, i.e., one not in the process of maturation, and, as was expected, would retain calcium granules within the protective membrane of each spherule. Partial sclerotization may occur at the time a plasmodium has completed its trophozoitic activity and is in the process of sporangial formation.

From the above review of the literature relative to the structure of the sclerotium it is evident that there is yet a need for further study of this resting stage in the life history of the Myxomycetes, especially since observations on the nuclei have not been reported by the above students. Accordingly, a series of experiments were devised to determine the structure of the sclerotium and the behaviour of the protoplasm and the nuclei of each spherule upon the

reactivation of the sclerotium. The results of these experiments are embodied in this paper.

MATERIALS AND METHODS

SCLEROTIUM REACTIVATION

TIME	DATE	SMEAR #	REACTIVATION TIME (min)
1730 hours	3/5/50	1	0
1755 hours	3/5/50	2	25
1825 hours	3/5/50	3	55
1910 hours	3/5/50	4	100
2000 hours	3/5/50	5	150
2015 hours	3/5/50	6	165
2030 hours	3/5/50	7	180
2050 hours	3/5/50	8	200
2125 hours	3/5/50	9	235
2200 hours	3/5/50	10	270
2215 hours	3/5/50	11	285
2300 hours	3/5/50	12	330
2325 hours	3/5/50	13	355
2350 hours	3/5/50	14	380
0010 hours	3/6/50	15	400
0030 hours	3/6/50	16	420
0125 hours	3/6/50	17	475
0155 hours	3/6/50	18	505
0240 hours	3/6/50	19	550
0320 hours	3/6/50	20	590
0405 hours	3/6/50	21	635
0435 hours	3/6/50	22	665
0510 hours	3/6/50	23	700
0545 hours	3/6/50	24	735
0655 hours	3/6/50	25	805
1055 hours	3/6/50	26	1045
1055 hours	3/6/50	27	1045

TABLE I

Text Figure 1

Materials and Methods

An excellent sclerotium of Physarella oblonga Morgan was collected at Gayton coal mine in Henrico County in February, 1950, under the bark of a large fallen oak. This material was brought into the laboratory and kept for a period of ten days at a temperature of five degrees centigrade in a tight container.

A portion of this sclerotium was broken into very small fragments and sowed in a petri dish containing sufficient water to wet the material. Smears of this material were made at intervals of time as set forth in text figure 1, were fixed using the smear technique suggested by Johansen (19) with Taylor's modified Karpechenko fluid (19). The constituents of this fixative were kept in separate tight bottles and were mixed (in appropriate proportions) as required.

Allen's modification (P.F.A_3) of Bouin's solution (28) was also tried but did not give as good results as did theKarpechenko fluid.

After fixation for four to six hours, slides

were washed thoroughly in slowly running tap water, rinsed in distilled water, mordanted in four per cent iron alum for about one hour and stained in hematoxylin for one to twenty-four hours. Slides were removed from the hematoxylin and washed in running tap water for about ten minutes, passed through distilled water and destained with two per-cent iron alum until the nuclei were easily distinguishable. After destaining slides were washed in running tap water for twenty minutes, rinsed in distilled water, transferred to a graded series of alcohol and water, then through an alcohol-xylene series to absolute xylene and mounted in neutral balsam.

Delafield's hematoxylin (19) was also used and proved satisfactory as far as facility of counting nuclei is concerned but does not give the niceties of nuclear stain equivalent to the Heidenhain method.

Another portion of this sclerotium collected at Gayton coal mine in Henrico county, was divided into sixty-five approximately equal portions and each was placed in the center of a petri dish containing sterile three percent agar medium. It may be noted here that numerous reactivations were induced on

SCLEROTIUM REACTIVATION

TIME	DATE	SCLEROTIUM #	REACTIVATION TIME (min)
1700 hours	3/5/50	0	0
1710 hours	3/5/50	1	10
1750 hours	3/5/50	2	50
1825 hours	3/5/50	3	85
1915 hours	3/5/50	4	135
2000 hours	3/5/50	5	180
2015 hours	3/5/50	6	195
2030 hours	3/5/50	7	210
2050 hours	3/5/50	8	230
2125 hours	3/5/50	9	265
2140 hours	3/5/50	10	280
2200 hours	3/5/50	11	300
2210 hours	3/5/50	12	310
2235 hours	3/5/50	13	335
2305 hours	3/5/50	14	365
2325 hours	3/5/50	15	385
2350 hours	3/5/50	16	410
0010 hours	3/6/50	17	430
0035 hours	3/6/50	18	455
0105 hours	3/6/50	19	485
0110 hours	3/6/50	20	490
0130 hours	3/6/50	21	510
0230 hours	3/6/50	22	570
0245 hours	3/6/50	23	585
0320 hours	3/6/50	24	620
0340 hours	3/6/50	25	640
0355 hours	3/6/50	26	655
0405 hours	3/6/50	27	665
0430 hours	3/6/50	28	690
0505 hours	3/6/50	29	725
0530 hours	3/6/50	30	750
0540 hours	3/6/50	31	760
0605 hours	3/6/50	32	785
0630 hours	3/6/50	33	810
0655 hours	3/6/50	34	835
0725 hours	3/6/50	35	Discard
0750 hours	3/6/50	36	890
0825 hours	3/6/50	37	925
0830 hours	3/6/50	38	930
0910 hours	3/6/50	39	970
0940 hours	3/6/50	40	1000
1000 hours	3/6/50	41-44	Discard
1020 hours	3/6/50	45	1040
1035 hours	3/6/50	46	1055
1550 hours	3/6/50	47	1610
1620 hours	3/6/50	48	1680
1630 hours	3/6/50	49	1710
1710 hours	3/6/50	50	1810
1710 hours	3/6/50	51	1810
1735 hours	3/6/50	52	1835
0915 hours	3/7/50	53	2415
0920 hours	3/7/50	54	2420
1115 hours	3/7/50	55	2535
0025 hours	3/8/50	56	3325
2030 hours	3/8/50	57	4530

TABLE II

Text Figure 2

filter paper substratum but sectioning of the material was impossible because of the many cellulose fibres permeating the fixed material. Agar did not afford this difficulty.

Petri dishes were flooded with Taylor's modified Karpechenko fluid at intervals of time during the reactivation cycle as set forth in text figure 2. Time of fixation was about six hours. Fixative was made up fresh at the beginning of this experiment and a new batch was made at the time specimen #21, text figure 2, was killed.

At the end of fixation time, fixative was poured from the petri dish and tap water was added and changed several times during a half hour period. From water all specimens were run up to xylene through graded alcohol-water and alcohol-xylene series and infiltrated with a mixture of 89% paraffin, 10% beeswax and 1% lanolin (14).

For reasons of economy and efficiency, after the fixative had been removed from the petri dishes, specimens were transferred along with a portion of the agar substratum to small flat bottomed 2 ml vials. Liquids were transferred using capillary pipettes.

Preliminary infiltration of paraffin was accomplished in a petri dish filled with the paraffin-beeswax-lanolin mixture, in which as many as fifteen specimens could be handled at one time. Identity of specimen was easily handled by marking its numerical designation on the outside of the petri dish. In order to get a sufficient number of specimens to preliminary infiltration at the same time it was necessary to have them pile up in seventy per cent alcohol. This infiltration time was approximately one hour under an ordinary goose neck lamp. The melting point of the paraffin used was 52° C.

Final infiltration was done in paper boats in a DeKhotinsky oven at 60° C for several hours. The paraffin mixture was changed four times. Infiltration in bond paper boats is very satisfactory since the mixture diffuses out through the paper into a container and fresh paraffin is added as required.

Sectioning of material was done at five microns using a Spencer rotary microtome whose calibration was determined to be within 0.7 micron. These sections were affixed with Mayer's adhesive (19) on clean slides using the alcohol burner method.

Completed sections were mounted in balsam or Euparal. The latter intensifies hematoxylin stains and renders the nucleus clearer than does balsam.

The iodine, potassium-iodide-sulphuric acid method (19) was used to test for cellulose.

Sudan IV (19) in 70% alcohol was employed to ascertain the presence or absence of fatty substances.

The remaining portions of the sclerotium collected in February, 1950, were placed upon filter paper in standard petri dishes which contained portions of the organism's natural substratum, wetted with tap water and fed pulverized rolled oats as required. Several of these plasmodia, which had reactivated from the original sclerotium, were put in paper lined battery jars containing bark or wood of oak and fed rolled oats.

The Feulgen nucleal reaction (19) was carried out in coplin jars which had been washed thoroughly with tap water and detergent, rinsed and cleaned with potassium dichromate, sulphuric acid solution, rinsed in tap wa ter and finally rinsed in several changes of distilled water. After cleaning, these jars were

inverted over absorbent paper towels until dry. All volumetric and filtering apparatus was cleaned in potassium dichromate cleaning solution and rinsed in distilled water.

The Feulgen method (19) is as follows: Five hundred milligrams of 95% basic fuchsin, certified for use as a Feulgen stain, was dissolved by pouring over it one hundred ml of boiling distilled water and agitating considerably. After cooling this solution to fifty degrees centigrade and filtering, five hundred milligrams of potassium metabisulphite and ten ml of 1/N HCl were added. The stain was then agitated vigorously, placed in a dark bottle for twenty-four hours to allow reduction of the basic fuchsin to leuco-fuchsin.

Slides, containing specimens embedded in paraffin, were brought down through the xylene-alcohol series and the alcohol-water series to distilled water. From distilled water they were taken to 1/N HCl for a quick rinse, then placed in fresh 60° C 1/N HCl for fourteen minutes which seems to give the best results for this material.

Slides were transferred from the sixty degree cen-

tigrade 1/N HCl to cold 1/N HCl, to distilled water, and then into the Feulgen stain. Time of staining was eight hours. They were removed from the staining solution and passed through three solutions, each containing 100 ml of water, 5 ml of 10% potassium metabisulphite and 5 ml of 1/N HCl. Time of exposure to each of these three solutions was ten minutes. After staining, slides were taken back to xylene and mounted in balsam

Experimentation was carried out in a laboratory in which the temperature fluctuated from 21 to 24° centigrade during the period February to June 1950.

Measurements were made with a 97X, N.A. of 1.25 or a 100X, N.A. of 1.3 oil immersion lens and a 10X occular. The precision here is 0.7 microns. Photographs were taken with Panatomic-X or Contrast Process Panchromatic film. The latter is undoubtedly superior as shown by comparative photomicrographs.

The method of dissolving calcium granules by using acetic acid as suggested by Brandza (5) was not successful for the sclerotium of Physarella oblonga. Potassium dichromate sulphuric acid solution removed these granules in one or two minutes, depending upon

the stage of reactivation of the sclerotium.

OBSERVATIONS

Observations

The sclerotium of Physarella oblonga Morgan consists of numerous spherical to polyhedral spherules, measuring from seventeen to twenty-one microns in diameter which are bound together by a cement substance which is continuous with the cortex of the sclerotium (Plates 1 and 2). The cortex is occasionally reticulated (Plate 3). This reticulation is similar to the reticulation frequently encountered between spherules within the sclerotium (Plate 4) of this species, and is somewhat comparable to the filamentous trabeculae figured by Brandza (5) for Didymium difforme. In D. difforme however the spherules are widely separated and the cement substance is outstanding whereas in Physarella oblonga the spherules are closely bound together and a reticulation of the cement substance does not exist between all spherules.

Spherule walls are about one micron thick and do not give any detectable reaction to the iodine, potassium-iodide-sulphuric acid test for cellulose but remain clear and transparent. They frequently stain with Delafield's hematoxylin however and are

assumed to be some form of cellulose mainly because several investigators have found this region to be some form of cellulose while studying other species.

Often there are observed at the junction of several spherules, masses of irregularly shaped to spherical granules which measure one micron in diameter and stain intensely with hematoxylin (Fig. 1, Plate 5). In some cases these granules remain within the empty spherule membranes after the spherules have germinated and the plasmodium is collecting together outside of the empty spherule containers (Figs. 2, Plate 5), whereas in other cases they are seen incorporated in the active macro-plasmodium, although they are still embedded in a portion of the cement substance and spherule wall of the original sclerotium. In any case it is seen (Figs. 1 & 2, Plate 5) that the diameter of these granules does increase during the reactivation cycle. They have not been observed to disperse in the plasmodium and are considered to be plasmodic wastes. It is noted however that their size is much like that of the mitochondria.

Spherules containing all of the constituents en-

countered in germinating spherules are often found in the moving plasmodium but reactivation of these spherules has not been observed. On the contrary, there is evidence that they are used for food by the reforming plasmodium. They give the appearance of partially digested masses closely ressembling disintegrating spherules.

Rarely, in the cortex of Physarella oblonga are found cysts (Plate 3) measuring seven microns in diameter as well as granules which stain intensely with hematoxylin. These granules are similar to those found between spherules deep in the sclerotium.

Within the spherule wall is the multinucleated protoplast which upon germination collects together inside of the cortex with numerous other protoplasts to form the plasmodium. The growth of a single protoplast into such a plasmodium has not been observed although numerous attempts were made to isolate single spherules in hanging drops by dilution and to transfer these isolated protoplasts to a suitable medium and watch for the germination and development of a large plasmodium. Failure to accomplish this is attributed to the techniques involved or possibly to the

		POSITION A			POSITION B			POSITION C			POSITION D			POSITION E			MEANS	N_p
		Spherule			Spherule			Spherule			Spherule			Spherule				
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3		
SMEAR #1	N_d	2.8	2.8	3.5	3.5	2.8	3.5	2.8	3.5	2.8	2.8	3.5	2.8	2.8	3.5	2.8	3.1	
	S_d	23.8	24.5	19.6	15.4	20.3	22.4	17.5	17.5	21.0	22.4	20.3	21.7	17.5	23.8	18.2	20.4	
	$N_\#$	9	11	3	4	6	8	10	9	11	14	5	10	9	12	7	8.9	0.0352
SMEAR #2	N_d	3.5	2.8	2.8	3.5	2.8	2.8	3.5	2.8	2.8	xxx	xxx	xxx	xxx	xxx	xxx	3.0	
	S_d	20.3	17.5	18.2	16.9	18.9	18.9	18.2	15.4	19.2	xxx	xxx	xxx	xxx	xxx	xxx	18.4	
	$N_\#$	10	10	12	10	9	6	7	9	10	xxx	xxx	xxx	xxx	xxx	xxx	9.4	0.0422
SMEAR #4	N_d	3.5	3.5	2.8	2.8	2.8	2.8	3.5	2.8	2.8	3.5	2.8	3.5	xxx	xxx	xxx	3.1	
	S_d	17.5	14.0	18.9	16.1	18.2	16.2	18.2	16.1	17.5	16.1	17.5	16.1	xxx	xxx	xxx	17.0	
	$N_\#$	9	4	6	6	8	13	7	8	9	7	8	6	xxx	xxx	xxx	7.6	0.0519
SMEAR #9	N_d	3.5	3.5	2.8	3.5	2.8	2.8	3.5	3.5	xxx	3.5	2.8	3.5	3.5	3.5	3.5	3.3	
	S_d	20.3	14.7	21.0	21.0	17.5	21.0	20.3	15.4	23.0	17.5	20.3	16.1	21.0	17.5	14.7	19.1	
	$N_\#$	9	4	5	9	10	5	8	4	xxx	9	6	9	6	6	7	6.8	0.0381
SMEAR #10	N_d	3.5	3.5	2.8	2.8	2.8	3.5	3.5	xxx	xxx	3.5	2.8	xxx	xxx	xxx	xxx	3.2	
	S_d	17.5	21.0	22.4	16.8	19.6	21.0	17.5	xxx	xxx	21.0	19.6	xxx	xxx	xxx	xxx	19.7	
	$N_\#$	6	8	10	8	7	8	9	xxx	xxx	12	10	xxx	xxx	xxx	xxx	7.8	0.0336
SMEAR #11	N_d	2.8	3.5	2.8	3.5	2.8	4.2	2.8	3.5	3.5	3.5	3.5	3.5	2.8	2.8	xxx	3.3	
	S_d	16.1	17.5	17.5	21.0	21.0	21.0	19.6	18.9	16.1	15.4	17.5	17.5	16.9	17.5	xxx	18.3	
	$N_\#$	6	7	8	9	11	3	11	10	7	6	10	1	8	10	xxx	7.6	0.0427
SMEAR #12	N_d	2.8	3.5	2.8	3.5	3.5	3.5	3.5	3.5	2.8	3.5	3.5	3.5	3.8	2.8	2.8	3.2	
	S_d	21.0	21.0	18.9	21.7	18.9	21.7	18.9	17.5	16.1	20.3	21.0	21.0	18.2	18.9	21.7	19.8	
	$N_\#$	11	9	6	6	7	9	6	8	2	8	8	6	10	6	8	7.3	0.0316
SMEAR #13	N_d	2.8	2.8	2.8	3.5	3.5	3.5	3.5	3.5	2.8	2.8	2.8	2.8	3.5	3.5	2.8	3.1	
	S_d	21.0	21.0	20.3	20.3	19.6	23.1	17.5	20.3	21.0	18.9	16.8	21.0	21.0	23.1	20.3	20.3	
	$N_\#$	5	8	6	8	4	12	9	12	10	11	7	13	8	9	6	9.5	0.0336
SMEAR #14	N_d	2.8	3.5	2.8	3.5	3.5	2.8	3.5	2.8	2.8	2.8	3.5	2.8	3.5	2.8	3.5	3.1	
	S_d	17.5	17.5	16.9	21.0	20.3	24.5	20.3	14.7	21.0	20.3	16.8	18.9	21.0	16.8	18.9	18.4	
	$N_\#$	4	7	2	11	12	13	9	6	11	8	2	8	12	7	10	8.5	0.0391
SMEAR #15	N_d	2.8	2.1	2.8	2.8	3.5	2.8	2.8	3.5	2.8	2.8	3.5	2.8	2.8	3.5	3.5	3.0	
	S_d	18.9	14.0	20.3	21.0	20.3	21.0	21.0	18.9	21.0	21.0	18.9	17.5	21.7	17.5	24.5	19.6	
	$N_\#$	8	4	9	12	4	10	8	10	14	8	7	14	9	6	11	8.9	0.0321
SMEAR #16	N_d	2.8	3.5	2.1	3.5	2.8	3.5	2.8	3.5	3.5	3.5	2.8	2.8	3.5	2.8	2.8	3.1	
	S_d	24.5	17.5	18.9	21.0	21.0	21.7	16.1	21.0	21.0	17.5	21.0	20.3	21.0	21.0	18.9	20.2	
	$N_\#$	9	4	6	8	7	6	10	7	5	9	10	9	5	11	10	7.7	0.0312
SMEAR 18	N_d	2.8	2.8	2.8	2.8	3.5	2.8	2.8	2.8	3.5	2.8	2.8	3.5	2.8	3.5	3.5	3.1	
	S_d	22.4	18.2	17.5	18.2	21.0	14.0	24.5	18.2	19.6	20.3	20.3	26.6	26.6	25.2	19.6	19.9	
	$N_\#$	8	20	10	7	9	5	10	10	4	14	9	14	13	5	8	9.7	0.0407
SMEAR 19	N_d	3.5	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	3.5	2.8	2.8	2.9	
	S_d	19.6	20.3	19.6	17.5	20.3	17.5	16.8	18.9	18.9	16.1	14.7	18.9	18.2	18.4	18.2	18.4	
	$N_\#$	6	7	12	11	12	12	8	9	10	10	3	8	6	5	7	8.4	0.0377
SMEAR 21	N_d	2.8	3.5	2.8	3.5	2.8	2.8	2.8	3.5	2.8	2.8	2.8	2.8	3.5	2.8	2.8	3.0	
	S_d	21.0	18.2	18.2	17.5	20.3	16.8	24.5	20.3	23.4	18.9	22.4	19.6	16.8	14.7	16.8	19.2	
	$N_\#$	16	2	5	6	10	8	13	5	12	2	11	16	6	5	9	8.4	0.0331

LEGEND: N_p- Nucleoplasmic ratio; N_d-Nuclear diameter; $N_\#$-Nuclear number; S_d-Spherule diameter

Text Figure 3.

inability of this protoplast to compete with other organisms since these cultures were not free from bacteria.

Spherule and nuclear diameters were measured and the number of nuclei per spherule was determined from fourteen of the twenty-seven specimens listed in text figure 1 (opposite page 9). This data is contained in text figure 3. Examination of this table brings out the following points of interest, namely that each spherule protoplast contains from one to twenty nuclei although these extremes are rare and a mean nuclear number count per spherule of eight.

Numerous yellow granules mask many aspects of the spherule protoplast, such as mitochondria, fat globules and ramifications of the reticulated cytoplasm but offer little difficulty to general nuclear observations because of the clear region which invariably surrounds the nucleus. These granules measure about one micron in diameter and show considerable variation in size. Their diameters have not been followed through the reactivation cycle but they are believed to increase slightly with absorption of water and upon germination.

Upon removing all of the chromatic granules with potassium dichromate-sulphuric acid solution which takes only several minutes and does not interfere with the hematoxylin stain reaction, there is easily seen in the majority of spherules, depending upon the stage of germination, a great many oval to spherical bodies which absorb hematoxylin strongly (Fig. 1, Plate 6). They become outstanding only in latter stages of reactivation.

The above mentioned oval to spherical bodies are apparently the mitochondria which were described by Cowdry (11) for several genera of Myxomycetes. It should be noted (Fig. 1, Plate 6) that these granules are concentrated in large numbers at the periphery of the spherule and are scattered and few in number in the interior. This organization is invariably found. Figure 2, plate 6 shows these granules in the reorganizing plasmodium. It is not uncommon to find wide expanses of plasmodium entirely free of these bodies. There is some evidence that they are not free in the cytoplasm but are suspended by delicate strands and give the appearance of being surround-

ed by a much lighter region than the adjoining cytoplasm. It is difficult to ascertain whether or not this phenomenon is due to shrinkage. The lack of observations on living material and the determination of relative coefficients of expansion and contraction of the cytosome with its plasma membrane; the nucleus and its constituents, and other portions of this material, by controlled fixation conditions have been in demand throughout this experimentation.

Cowdry (11) found mitochondria more abundant about the nuclei and around peripheral regions of vacuoles, however such could not be verified in this study. He did not believe them to be bacteria because they were never encountered in vacuoles and also were the first indication of impending spore formation when the organism was to fruit, in that they clumped together.

Sudan IV gives an intense reaction to indicate fatty substances stored in the spherule in the form of oval to spherical droplets, which vary in diameter from the lower limit of visibility to four or five microns. These droplets do not occur in all

spherules in quantity but are generally present throughout the sclerotium. Occasionally there are found spherules which are packed with apparently nothing but these substances. However careful consideration brings out at least one nucleus. These are the spherules most frequently encountered in the reorganizing plasmodium.

It has been observed by Cowdry (11) for the plasmodium and by Brandza (5) for the sclerotium that nuclei are lacking entirely in certain large regions of the plasmodium and occasionally in individual spherules. Spherules of Physarella oblonga have never been observed which lack nuclei altogether, although the variation of the nucleoplasmic ratio is tremendous for both sclerotium (Text fig. 3) and macroscopic plasmodium. From table 3 it can be seen that this variation in nucleoplasmic ratio exists from spherule to spherule.

The nucleus is the most interesting structure common to the various phases in the life history of the Myxomycetes because of its obvious inconsistency. For example, Jahn (17) found nuclear division of swarm cells of Stemonitis flaccida Lister, to be intra-

nuclear as far as he could determine. Furthermore the point of the intranuclear spindle contained a single granule, the centrosome, from which the flagellum was destined to arise. However, A. Lister (24) concluded that the nuclei of an active plasmodium multiplied by simple division after he had examined slides prepared from a plasmodium of Badhamia utricularis Berk., whereas J.J. Lister (26) found nuclei dividing by mitosis in the plasmodium of Badhamia utricularis which had been reactivated from a sclerotium. All stages were observed and recorded.

A. Lister (22) again, after numerous investigations with different species, put forth the belief that although nuclei in the plasmodium of B. utricularis had been observed to divide by mitosis by J. J. Lister in 1893, that when these nuclei are dividing in large numbers they do so by amitosis.

J. J. Lister (26) in 1909 concluded that nuclei in a macroscopic plasmodium may divide by mitosis but more often by amitosis.

Jahn (18) reported nuclear division again in the plasmodium of B. utricularis. His preparations were

not conducive to exact description of the process, however he found no indication of multiplication by simple division.

G. Lister (25) reported division by mitosis in slides prepared by J. J. Lister and injected the belief that probably the nuclei also divide by amitosis.

Wilson and Cadman (40) in their work on the cytology of the swarmers of Reticularia lycoperdon Bull., found typical mitotic division and referred to the centrosome formerly described by Jahn as the centroblepharoplast.

Schünemann (31) found metaphase, anaphase and telophase figures in a large plasmodium of Didymium nigripes. Some of these figures were surrounded by nuclear membranes.

Cotner (10) demonstrated that instead of a single centrosome or centroblepharoplast as believed by Jahn, and Wilson and Cadman that this single body was in reality a complex of granules.

Howard (15) searched for nuclear divisions in the plasmodium of Physarum polycephalum Schw., extensively in 1930 and was unable to ascertain mitosis or simple

division, although he took samples from a plasmodium every half hour for thirteen hours. He observed that plasmodia of this species increased in size from tiny fragments to areas covering as much as two square feet with the number of nuclei per unit volume apparently remaining constant.

This same investigator (16), again working with P. polycephalum, was able to work out mitosis in great detail. He was able to show that nuclear increase in the macroscopic plasmodium was by "geometrical progression." This division is completed within twenty to forty minutes and is intranuclear. According to J. J. Lister (26) this fact helps to establish the myxomycete relationship with the Protozoa. However because of the absence of asters and centrosomes, Howard points out that the nucleus of Myxomycetes "resembles in many respects the type found in vascular plants."

Sinoto and Yuasa (33) and Yuasa (41) have shown that there are two blepharoplasts in uniflagellated and biflagellated swarmers of several species of Myxomycetes.

Ellison (12) verified the presence of two blepharo-

plasts in uni- and biflagellated swarmers of Stemonitis ferruginea Ehrenb. and S. fusca Rost., although one of them was not easily recognizable in the latter.

In this study of Physarella oblonga the nuclei were found to vary from 2.8 microns to 3.5 microns in diameter in the sclerotium but may be smaller in the plasmodium if mitosis has just occurred.

The sclerotium of Physarella oblonga contains at least two types of nuclei, if staining reactions and size are suitable criteria for distinguishing them (Plates 4 & 7). There is no correlation between size and difference in staining capacity, for nuclei of all sizes may stain darkly or lightly with hematoxylin and the faint positive Feulgen nucleal reaction indicates desoxyribose nucleic acid in nuclei of both types.

Differentiation of the hematoxylin stain, in order to bring out the metabolic nucleus of the spherule invariably destains metabolic nuclei of reactivating spherules to such an extent that only the nucleoli are clearly visible. There is a definite gradient in staining capacity from the dark and almost black

nucleus of the intact spherule to the faint nuclei of the plasmodium. The nucleolus is well defined in the latter but the nucleoplasm and nuclear membrane are too faint to be seen clearly. Such a staining gradient with hematoxylin may be indicative of the varying physiological activity of the nucleus in these different stages during reactivation.

The nucleus is suspended in a cell-like structure by delicate strands (Plates 8 & 9). This organization is somewhat like the syncytial arrangements found in higher organisms and was first suggested by Camp (7) but he apparently did not recognize the definite irregular cell-like structure which contained the nucleus.

Brandza (5) had observed in reactivating sclerotia of many species such an organization and had termed these nuclei suspended by strands "amoebulae". His figures show amoebules with a wide range of variation in nuclear diameter whereas in this work the nucleus was found to be rarely outside the limits set forth in text figure 3, namely from 2.8 to 3.5 microns. Apparently Brandza considered amoebules as separate

individuals within the spherule protoplast.

The nucleolus which may vary from one large body to several smaller ones could be mistaken for mitosis if hematocylin were used and especially if only a few apparent mitotic figures were found. The nucleolus assumes many shapes and sizes and often is separated within a spindle-like region of the nucleus indicative of anaphase in mitosis (Fig. 1, Plate 10). These phases are not rare but seem to cover great areas of the reactivating sclerotium and all stages similar to mitosis up to late anaphase can be found. Prophase gives the greatest difficulty since the nucleolus may be in many tiny bodies which is similar to a thickening of the chromanemata and, without the Feulgen technique, might be interpreted as prophase.

Howard (16) has worked out mitosis in Physarum polycephalum thoroughly and found division to be intranuclear and without asters but with a well developed spindle. He did not find nuclei with more than five nucleoli for this species whereas eight to ten are not uncommon for Physarella oblonga (Fig. 1, Plate 7.).

The amoebule boundary, with few exceptions, is closely applied against the nucleus in the quiescent spherule. This boundary is practically indistinquishable from the nuclear membrane with which it is intimately bound by cytoplasmic processes which are only observed when the amoebule swells with imbibition of water and its boundary pulls away from the nucleus (Pl. 8; Pl. 9, Figs 1&2, and Pl. 10, Fig. 2).

It is indisputable that the individual spherules swell to some extent by rounding out into almost perfect spheres with water absorption (Plate 10, Fig. 3) when isolated one from the other. This smoothness of peripheral contour is not as obvious when the spherules are undisturbed in the sclerotium because of mutual pressure from other spherules. In general these structures are polyhedral in the resting sclerotium.

Upon germination the protoplast flows from one spherule through an opening in the connecting walls and joins with the protoplast of another spherule (Plate 8) or several coalesced protoplasts. It is not unusual to see many protoplasts fused together while they are still in their individual spherules

(Plate 11).

There is some evidence of pressure in the germination process (Plate 8; Plate 10, Fig. 4 and Plate 12, Fig. 1) in that the spherule wall is folded back into the spherule into which the protoplast from another spherule is flowing. Even though this has been observed numerous times, such an apparent violence of ge rmination could not be demonstrated with single spherules germinating in hanging drops. It is conceivable that because of pressure differential variation as regards isolated spherules and undisturbed sclerotial organizations the method is quite different.

Shrinkage of material is indicated in a reactivating sclerotium. This apparent distortion is due to a lack of protoplasm within the individual germinating spherules brought about by their contribution to the collecting plasmodium, which by this time has reformed and is outside the sclerotium (Plate 11), but still with numerous connections with individual spherules throughout the sclerotium. Reactivation (Plate 11) is centripetal and as well as could be determined, all germinating spherules are connected to-

gether. Germination is a smooth continuous process.

The plasmodium may take the form of numerous processes radiating from the sclerotium and might seem to indicate that more than one plasmodium could escape from a sclerotium, however, by following the process through serial sections, it is seen that all processes from the sclerotium merge.

The plasmodium, when fully formed, moves away from the germinating point and leaves behind the distorted, torn and empty protoplast containers, the spherule walls (Plate 13).

DISCUSSION
and
CONCLUSIONS

Discussion and Conclusions

The amoebules of Brandza (5) are apparently not individuals incorporated in the spherule but represent the metabolic nucleus and the substance in which it is contained, a portion of cytosome. Numerous cytoplasmic processes are continuous with this envelope and the nucleus. These processes are in the form of delicate strands which may be the result of fixation distortions. A study of living material is required to determine whether these connections are actually strands or reticulated cytoplasm.

In the majority of instances in the spherule a cytoplasmic envelope surrounding the nucleus is not clearly seen because of shrinkage of the system during sclerotium formation. It is reasonable to expect that one would find quite a variation in the distance from the envelope to the nucleus. Such is the case, with a variation from beyond the precision of measuring devices used and outside the resolving powers of the optical system, to a distance of approximately one micron.

Spherules are bound together with a cement sub-

stance which is believed to aid in water conduction throughout the sclerotium, which undoubtedly already has a high affinity for water. A rapid conduction of water through the cement substance would facilitate control of reactivation to some extent and this is probably why the plasmodium does not escape in individual waves, but does so in one unit mass.

Prior to germination, mitochondria (Plate 6, figs. 1 & 2) collect in large numbers near the wall of the spherule. They apparently play an important part in weakening the spherule wall to such an extent that the protoplast which meanwhile has taken on considerable quantities of water and has thereby increased internal pressure is able to burst through with some violence, folding back the spherule wall (Plate 10, fig. 4 and Plate 12, fig. 1). Each spherule has to overcome two spherule walls to germinate except those at the periphery of the sclerotium, unless the dissolution of the membrane in the region of breakthrough is a mutual process with each spherule digesting away its own wall and the one with the highest internal pressure would be the first to move by rushing

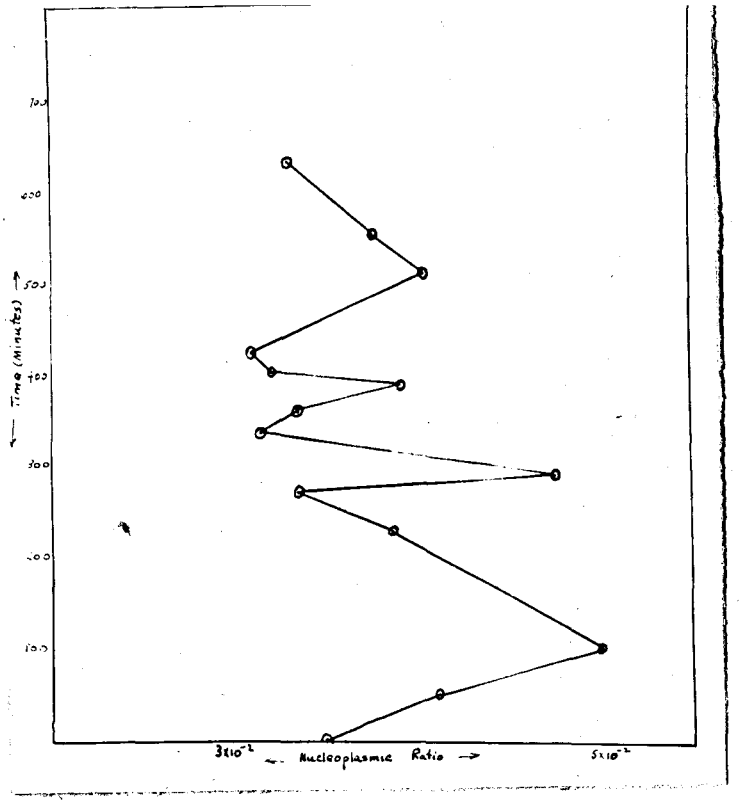
through the weakened container to join the protoplast on the lower side of the pressure differential.

Those spherules on the periphery of the sclerotium are restricted by only one such wall plus a wide cortex, many times thicker than the protoplast envelope (Plate 1). It is immediately suggested that the cortex of a sclerotium would necessitate germination and coalescence first within the restraining cortical substance. This is exactly what takes place (Plate 14) and accounts in part, for the control of plasmodium escape. The cortex is the last retaining wall to be punctured and then only in weakened portions. This is difficult to establish with unquestionable certainty because of the method of beginning this work, i.e., using small bits of a large sclerotium, which of course were lacking in cortex in many regions. It is believed that studies of entire sclerotia germinating would show formation of the plasmodium within the cortex to be the general condition. Occasional reticulation of the cortex may play an important part in sclerotial germination, by facilitating escape through this boundary.

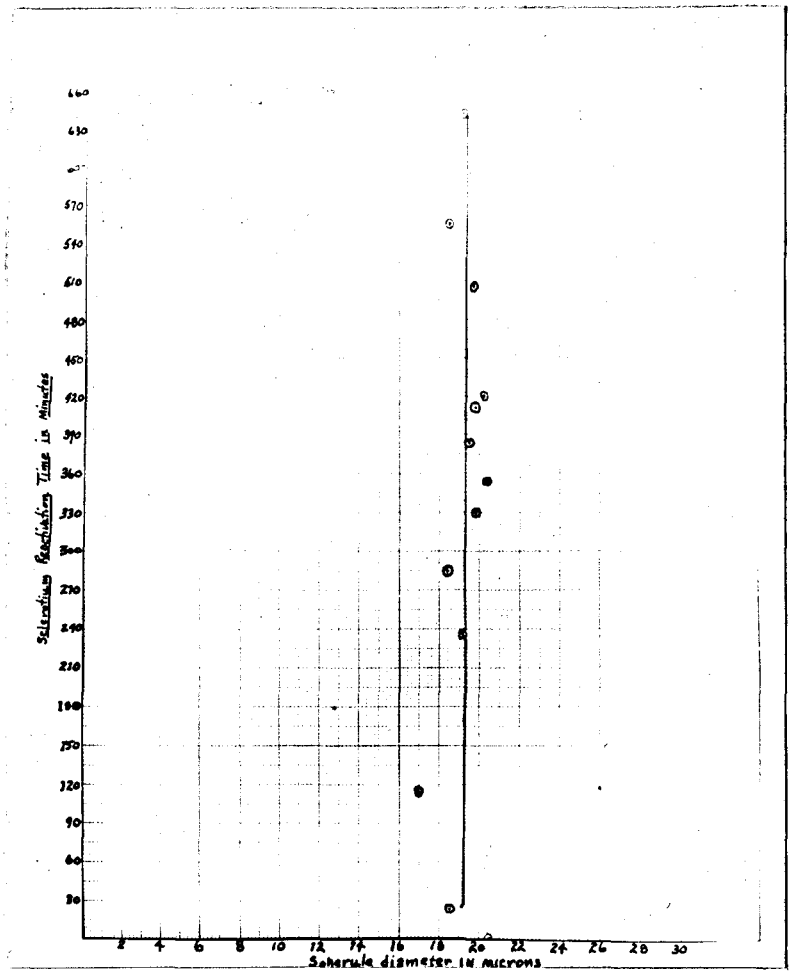
It is easily seen that there is a significant

physiological difference between the nuclei of quiescent spherule protoplasts and those of germinating ones, as mentioned previously. It is suggested that this difference in staining capacity was due to the concentration of desoxyribose nucleic acid in the nucleus which may be expected to be greater in the nuclei of the quiescent spherule whose metabolic activities were greatly reduced than in nuclei of the plasmodium which was preparing for extended activity and greatly accelerated metabolism.

The concentration of desoxyribose nucleic acid in the metabolic nucleus of Physarella oblonga is diffuse and gives only a faint reaction with the Feulgen technic. It was not possible to estimate in these small nuclei a difference in the desoxyribose nucleic acid concentration of the metabolic nucleus of the quiescent spherule and that of the interphase nucleus of the active plasmodium. Hematoxylin, however, shows a clear difference. The possibility that these dark staining nuclei are pycnotic must not be discarded. The "amoebulae" of Brandza might be discarded nuclei in vacuoles. If this were true then



Text Figure 4



Text Figure 5

the number of nuclei would be quite small for the plasmodium and spherule protoplasts. Spore formation does not support such a belief since the mortality rate of nuclei during this stage is low.

The question arose early in this work as to whether there were any nuclear divisions upon germination. The first attack upon this question was to investigate the nucleoplasmic ratio of the spherule during all stages of reactivation. If this ratio increased considerably then mitosis might have occurred. A curve, text figure 4, was plotted from the data summarized in text figure 3 and indicates that the karyoplasmic ratio actually decreases slightly during reactivation. This shows that the cytoplasm of a spherule increases slightly in volume whereas the volume of the nucleus remains essentially the same. Another curve, text figure 5, was also plotted from data summarized in text figure 3, and shows that the spherule diameter does increase slightly with time of reactivation. The shift in these curves is barely within precision of measurement and could be contributed to error.

Howard (16), however, had found that division in

the plasmodium covered wide expanses and was a geometric division with daughter nuclei considerably smaller than the original nucleus. This means that new nuclei would be less than the established range of diameter for the spherule. Detecting a change in the karyoplasmic ratio would of course be difficult. Furthermore, text figure 3 shows that the number of nuclei per spherule, which may vary from one to twenty, does not change during reactivation but has a constant mean. This is interpreted to show that nuclear division during or immediately after reactivation is not the general case. It is true of course that mitotic figures are found during this activity but they are rare. Several stages of mitosis are shown (Plate 12, figs. 2, 3, & 4), and are not intranuclear but take place within the amoebule envelope which is a cytoplasmic ground substance containing the nucleus.

Howard, Lister and Schuneman agree that division is intranuclear, although the last investigator found nuclear membranes surrounding only certain of the mitotic figures.

The Feulgen technique does not give a positive

reaction from the nuclear membrane out to the amoebule envelope.

From the above discussion it is evident that there is still need for more careful and detailed investigation of the nature and activity of the nucleus of the Myxomycetes.

SUMMARY

Summary

1. Germination of the sclerotium in Physarella oblonga is centripetal. The cortex hinders escape of the protoplasts in most regions, so that the plasmodium is well formed before it punctures the wide cortical region. This aids in the control of escape of the plasmodium.

2. There is some indication of enzymatic action and pressure factors combining to facilitate rupture of the spherule wall.

3. Nuclear divisions are rare during sclerotial reactivation and are not intranuclear.

4. The term "amoebulae" as first suggested by Brandza should be discarded since it is misleading. These organizations are considered to be nuclei embedded in cytoplasm with the cytoplasm giving the appearance of another wall.

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VITA

VITA

William Thomas Allman Jr. was born in December 26, 1916, at Richmond, Virginia.

Education: B.S., University of Richmond, 1940; Graduate work at University of Richmond, 1941; Army of the United States, September 1941- December 1945; Electrical Engineering study, Virginia Polytechnic Institute, Army Specialized Training Program, 1943.

General: Kellex Research Corporation, Manhattan Project - Atomic Energy, New York City Nash Building, 1944; Instrumentation supervision, Carbide and Carbon Chemical Corporation, K-25 Gaseous Diffusion Plant, Oak Ridge, Tennessee, 19d5-1949. Enrolled in Graduate School, University of Richmond in September 1949.

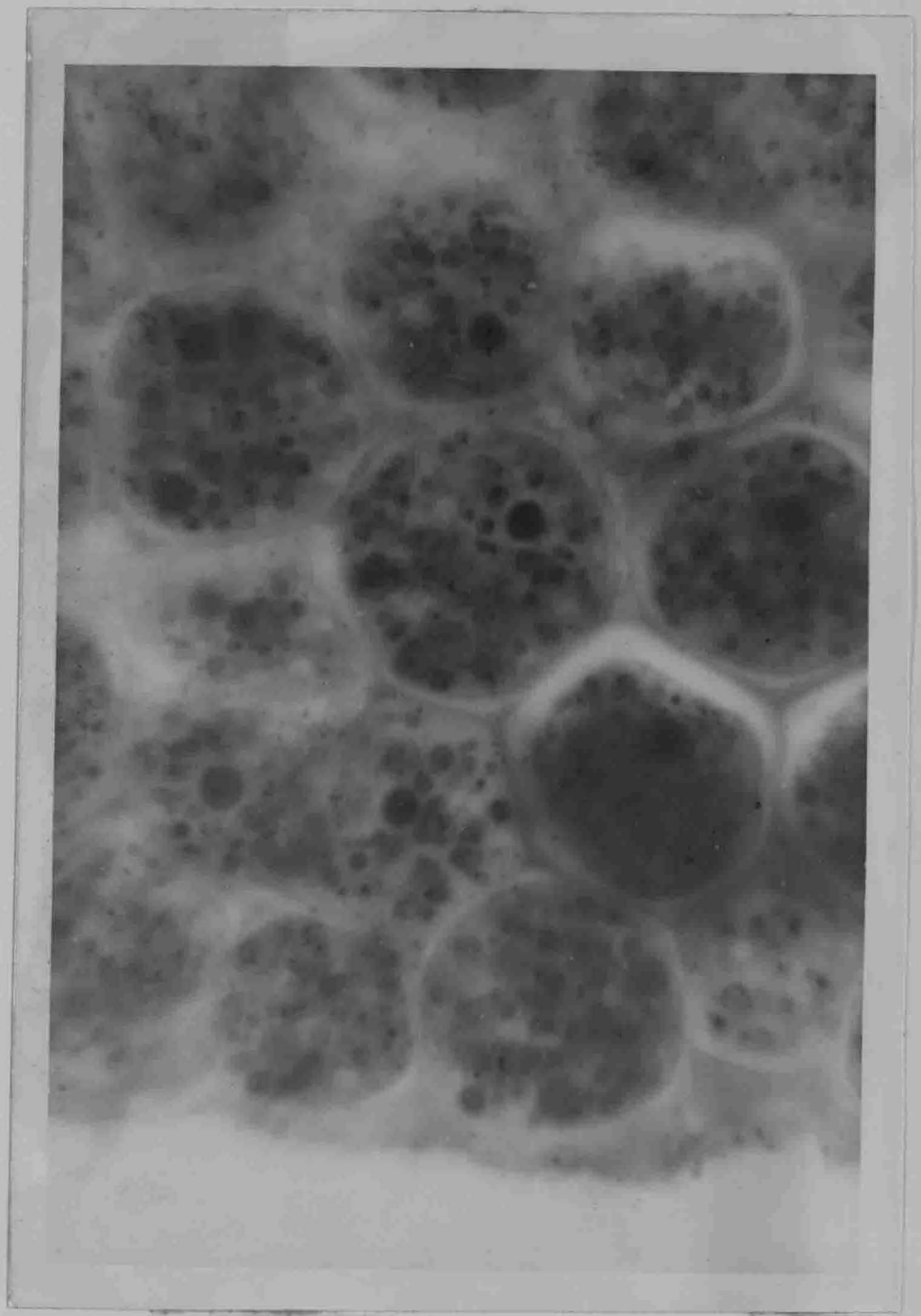
William Thomas Allman, Junior.
June 1950.

PLATES

Explanation for Plate 1:

Section (5 microns) through a resting sclerotium.

Note wide cortex at bottom of photograph, which is continuous with cement substance that binds spherules together.



←10μ→

PLATE 1

Explanation of Plate 2:

Resting sclerotium (section)
Cement substance is shown as dark black substance between spherules. (Middle of photograph)

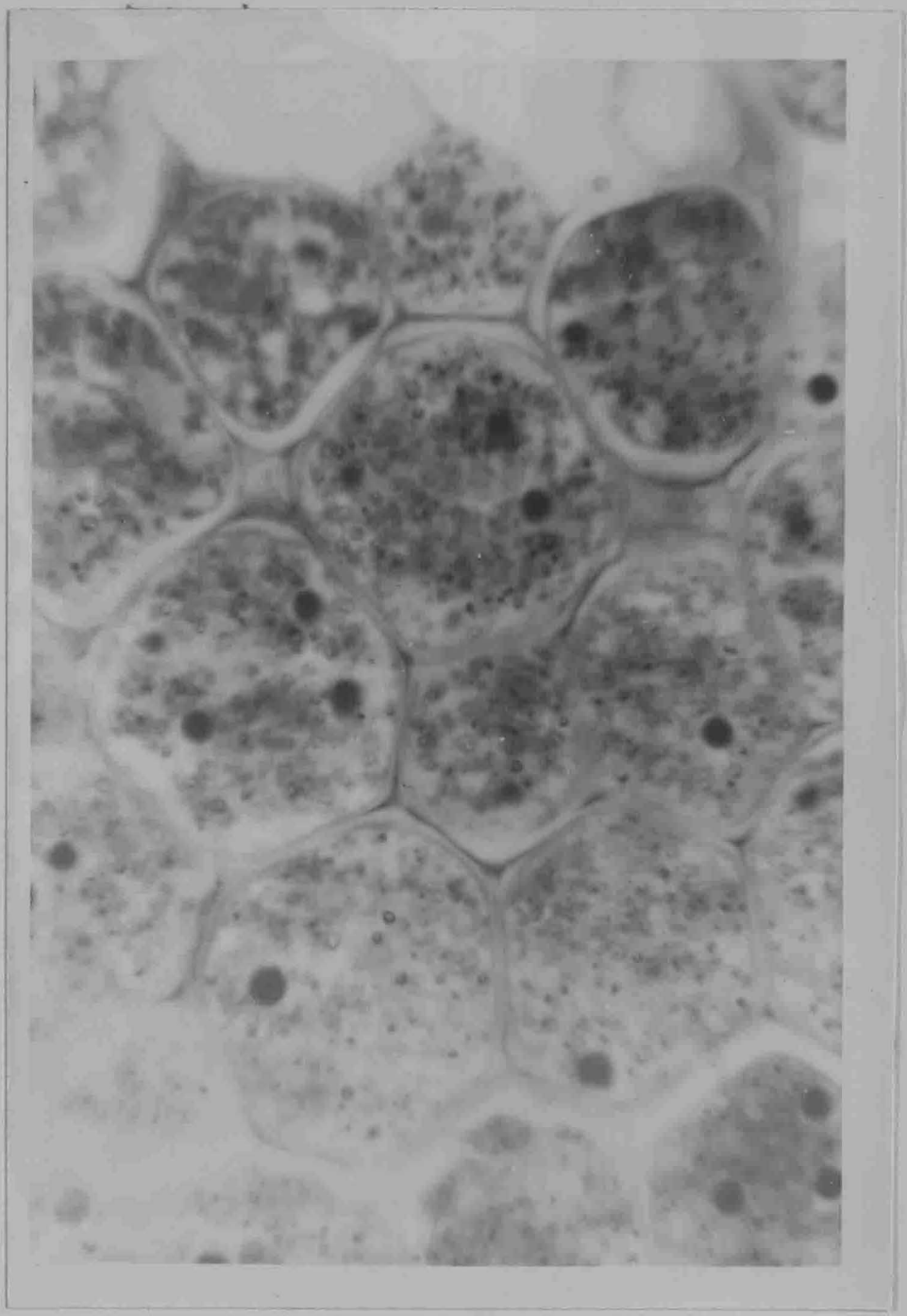


Plate 2

← 10μ →

Explanation of Plate 3:

a. Unidentified granules in cortex.

b. Sclerotium cortical region.

c. Reticulation of cortex.

d. Protoplast nucleus.

e. Cyst in cortex.

f. Reticulation from which fat droplet more than likely was removed.

g. Mitochondria.

h. Interior of sclerotium.

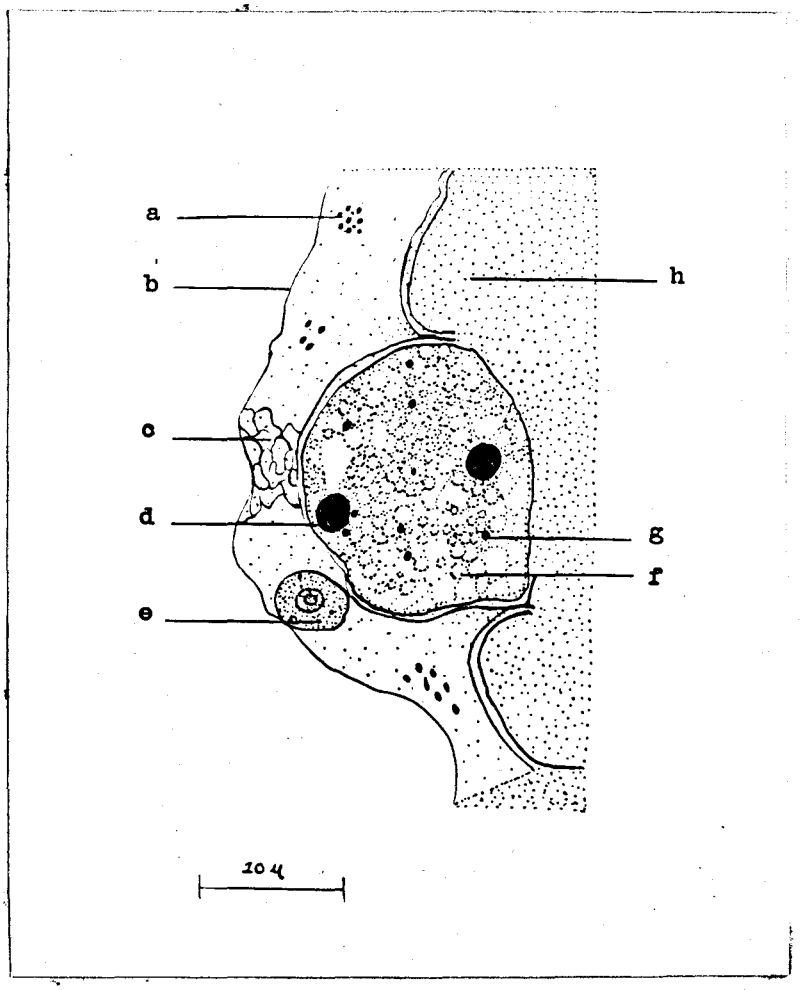


Plate 3

Explanation of Plate 4:

a. Boundary of spherule wall and protoplast membrane.

b. Mitochondria.

c. Nucleus.

d. Former fat droplet location.

e. Reticulation of cement substance.

f. Nucleus (note difference in staining).

g. Vacuole with inclusion.

h. Cement substance.

i. Unidentified mass of granules between spherules.

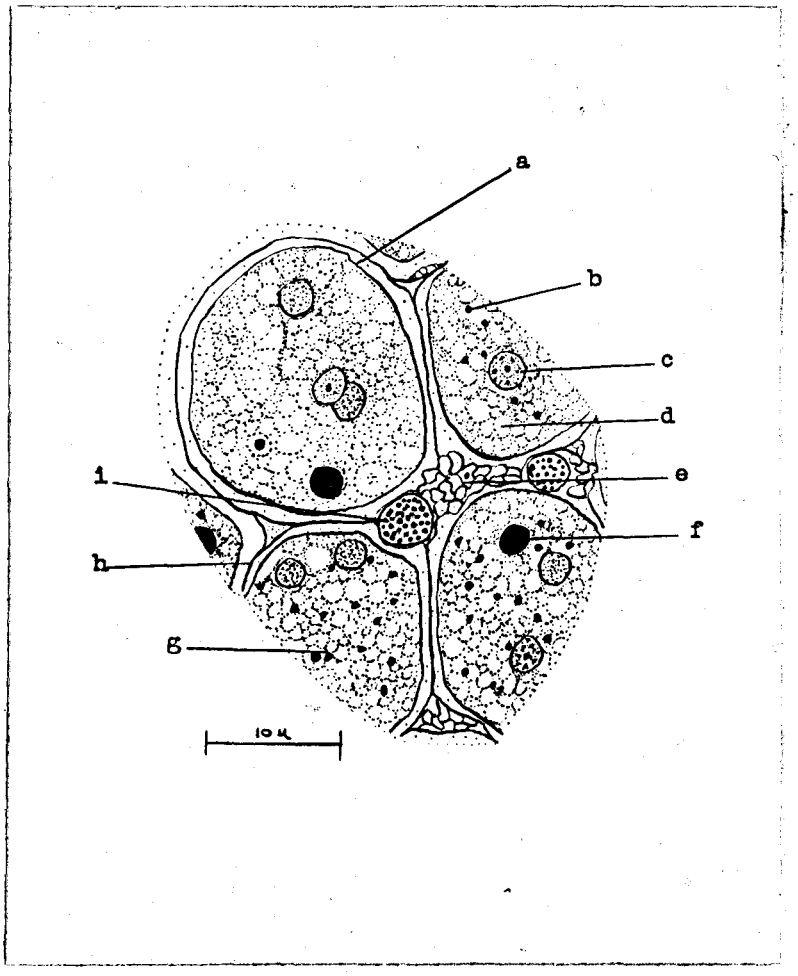


Plate 4

Explanation of Plate 5:

Fig. 1: Mass of unidentified granules between spherules. These granules enlarge with water uptake. (See Fig. 2). They are believed to be plasmodic wastes.

Fig. 2: Same granules as in Fig. 1 which have been left behind after reactivation. Note matrix in which they are contained. This matrix is apparently a part of the cement substance

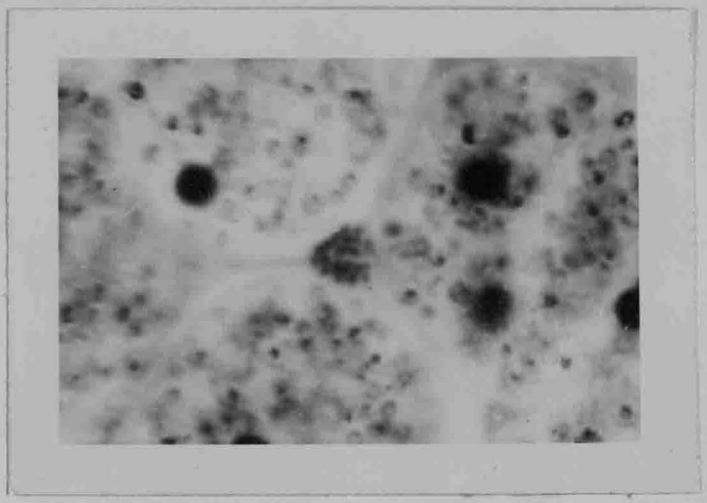


Figure 1

Figure 2

←— 10μ —→

Plate 5

Explanation of Plate 6:

Fig. 1: Early stage in reactivation. Notice absence of any apparent shrinkage and concentration of mitochondria at periphery of spherule. Protoplast is reticulated from loss of fatty substances, which were lost during the fixation process. Chromatic granules have been removed.

Fig. 2: A fully formed plasmodium showing scattered mitochondria.

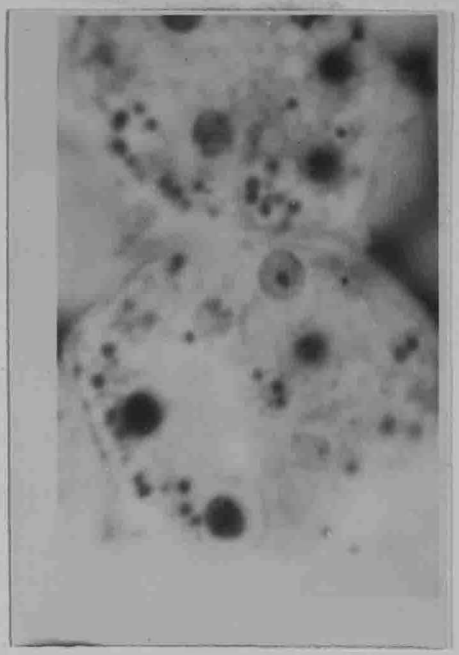


Figure 1

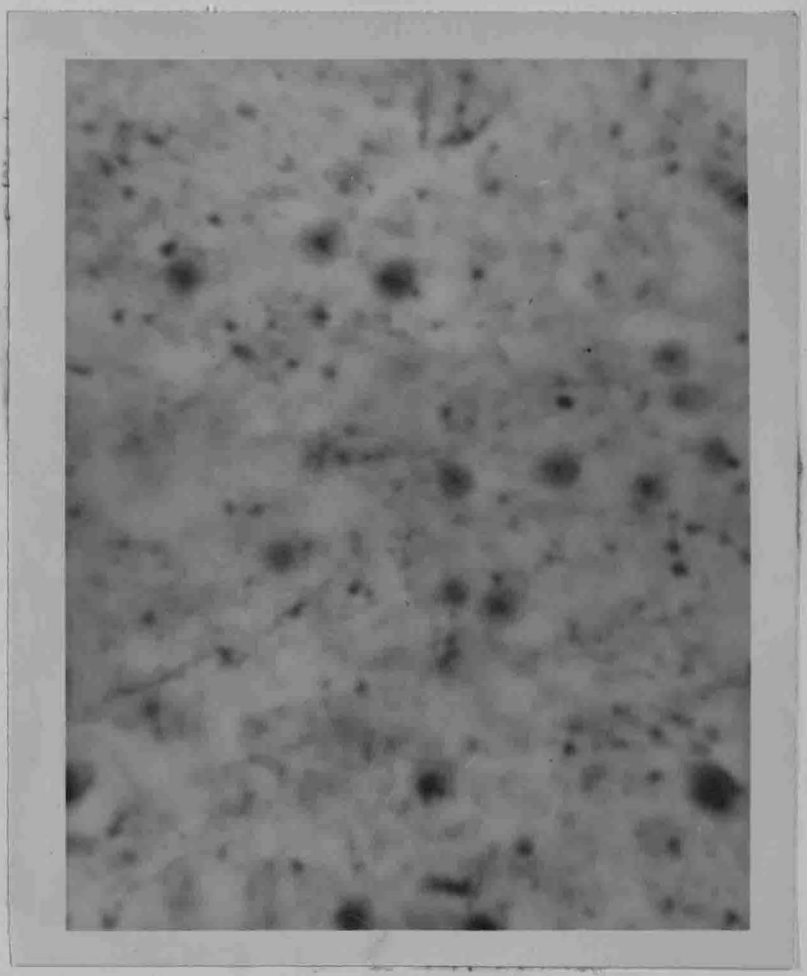


Figure 2

←10μ→

Plate 6

Explanation of Plate 7:

Fig. 1: A nucleus without a noticeable amoebule envelope. Ten small nucleoli are present in this nucleus.

Fig. 2: Early stage of reactivation. Note difference of nuclear staining capacity. This is the same photograph as Fig. 1, Plate 6.

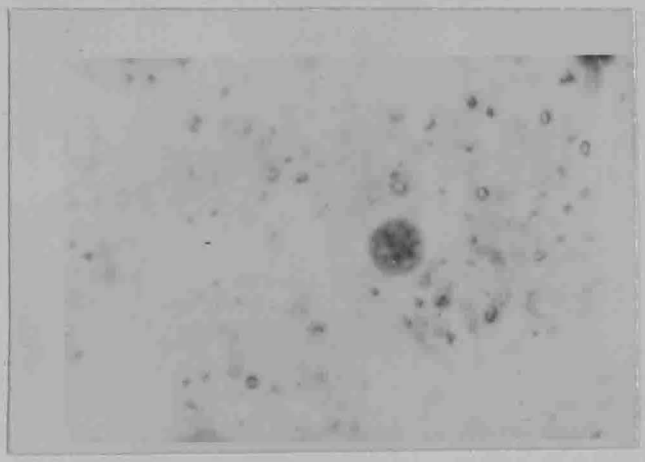
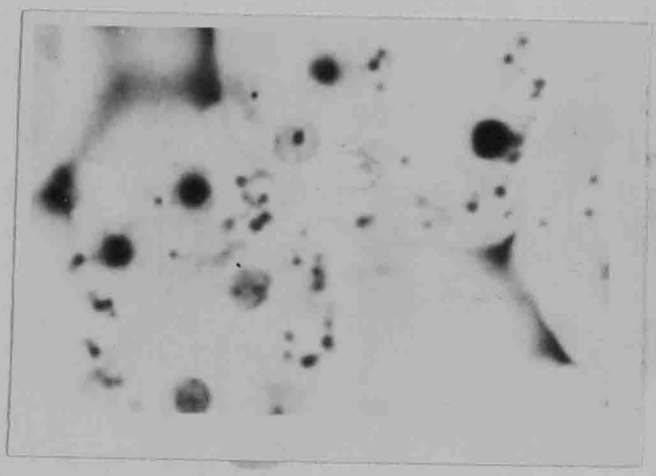


Figure 1



←— 10μ —→

Figure 2

Plate 7

Explanation of Plate 8:

Germinating spherules which together with many others are forming the plasmodium. Spherule walls are folded back into upper right spherule indicating some pressure is involved in germination. The magnification of this photograph was lost. It was taken with a 20X ocular and a 9.7X, 1.5 N.A. oil immersion lens.

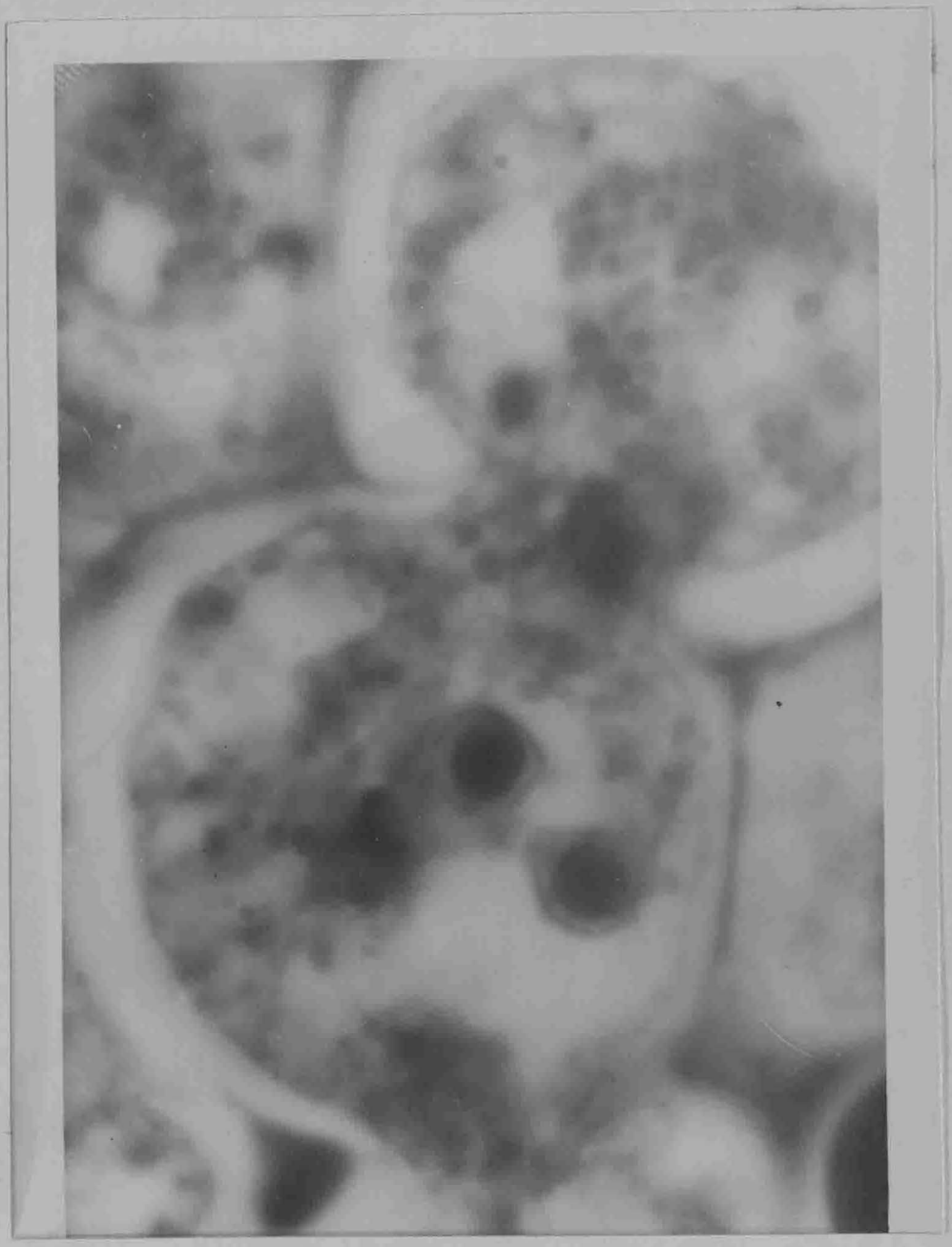


Plate 8

Explanation of Plate 9:

Fig. 1: This photomicrograph shows clearly the connections of the nucleus to envelope. Notice area around nucleus which is free from chromatic granules.

Fig. 2: Four nuclei, each of which is within a cytoplasmic envelope. All of these photographs are from reactivating spherules.

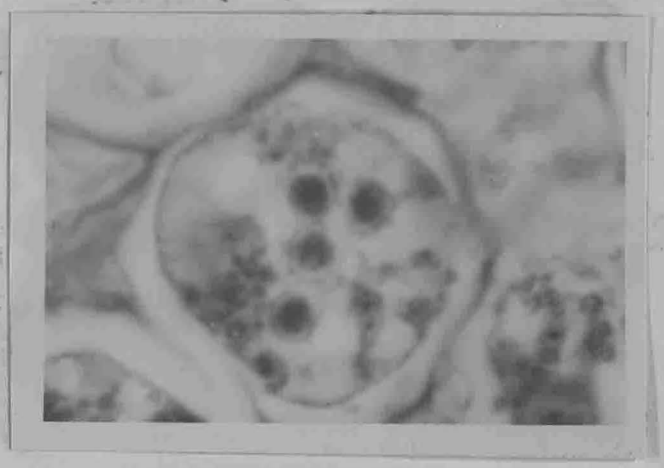
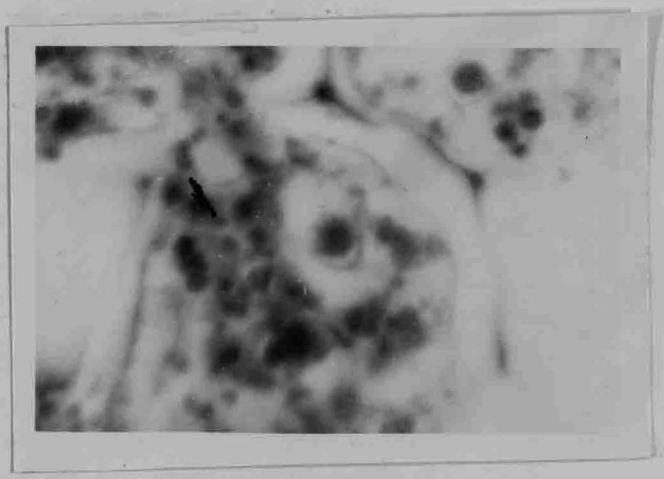


Figure 1



← 10μ →

Figure 2

Plate 9

Explanation of Plate 10:

Fig. 1: Note nucleus in center of photograph. There is the appearance of nuclear division. These nucleoli are contained within an elongated spindle, not separated in the middle. Feulgen shows these bodies to be nucleoli.

Fig. 2: Two nuclei within cytoplasmic envelopes. Note fusion of the envelopes. These nuclei are metabolic.(to show this would not bring out their amoebule envelope connection).

Fig. 3: A single spherule. This photograph was taken well into the germination cycle and is swollen and spherical. Nuclei are not shown clearly but can be made out near the top (2 of them). Some oil droplets can be observed which were not destroyed by fixation. From a smear preparation.

Fig. 4: Reactivation spherule (section). Note bulge into empty spherule at top of photograph. This expansion must be due to pressure. Apparently this protoplast had germinated at another point before this obvious bulge could rupture, however this could not be ascertained in serial sections. See Fig. 1, Pl. 12.

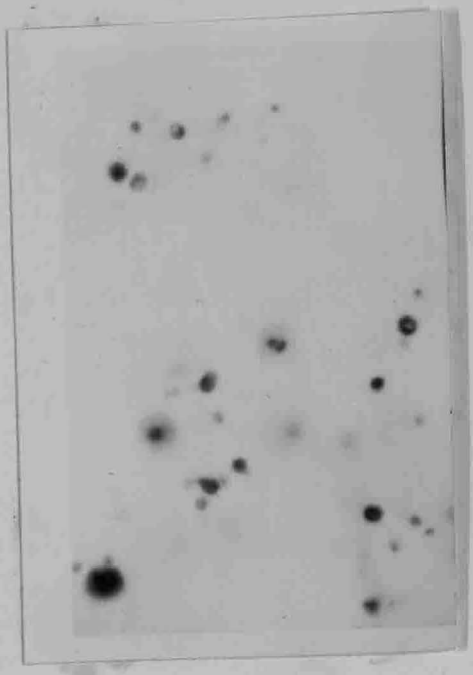


Figure 1

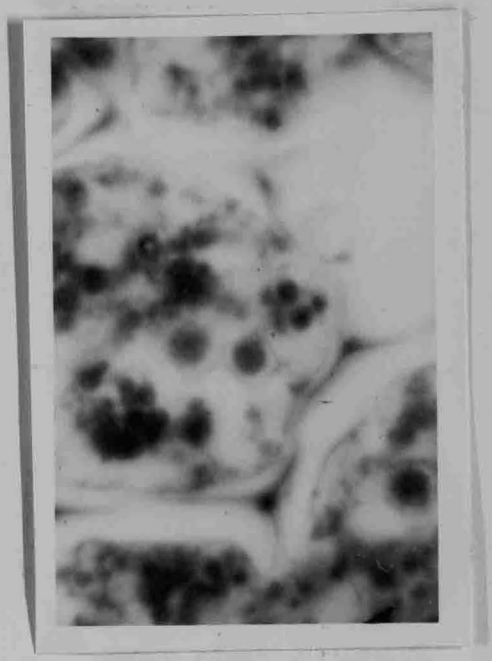


Figure 2

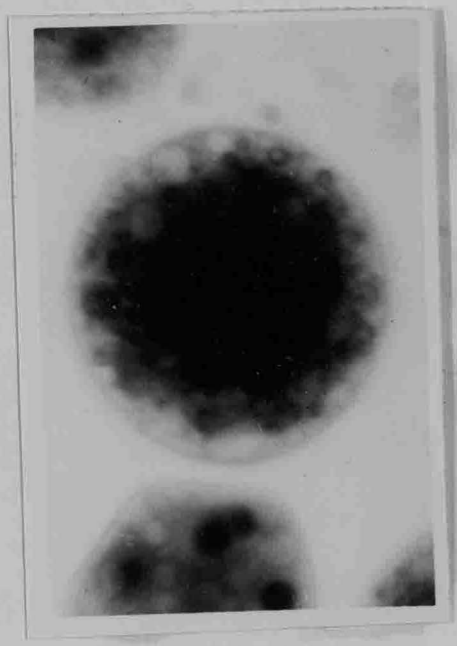


Figure 3

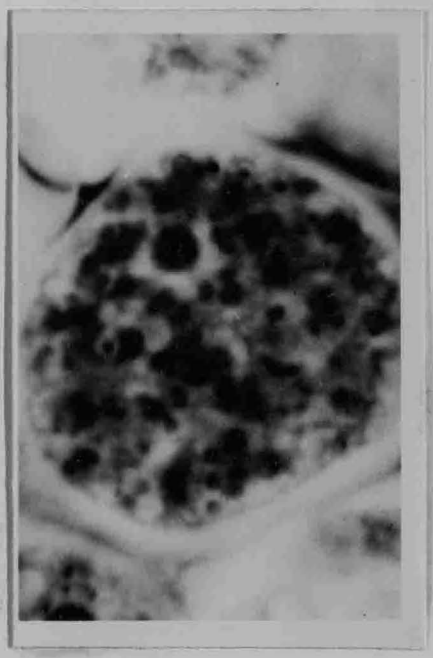


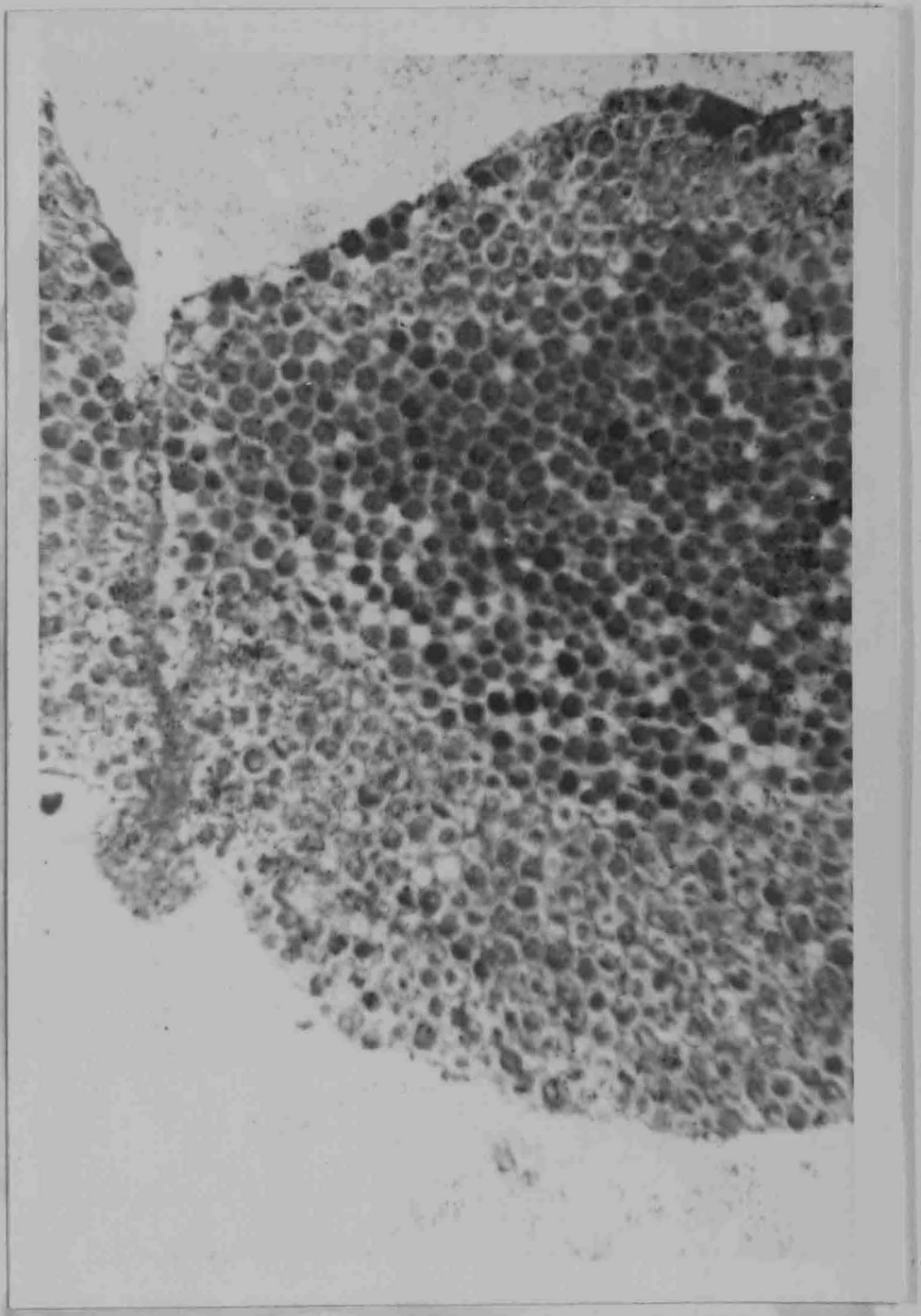
Figure 4

←10μ→

Plate 10

Explanation of Plate 11:

Reactivation of sclerotium is centripetal. Peripheral region is lighter because of the loss of protoplasm from individual spherules. This protoplasm has joined the escaping plasmodium shown at left of picture.



←10μ→

Plate 11

Explanation of Plate 12:-

Fig. 1: The bulge, as shown in Fig. 4, Plate 10, has broken out into an empty spherule. The two protoplasts in picture are joined. It is not unusual to find a spherule has germinated in more than one place. There is no reason that germination could not take place in two portions of the container simultaneously.

Fig. 2: Nucleus at bottom of photgraph is in prophase. Amoebule envelope is not obvious here but careful focusing shows it to be present. Note large, eccentric nucleolus.

Fig. 3: Nucleus at metaphase. Nuclear membrane is not present. Apparent membrane is cytoplasmic.

Fig. 4: Indistinct anaphase. Numerous photographs would not bring out this nucleus clearly. Cytoplasmic envelope is barely visible. Adjacent nuclei are in prophase.

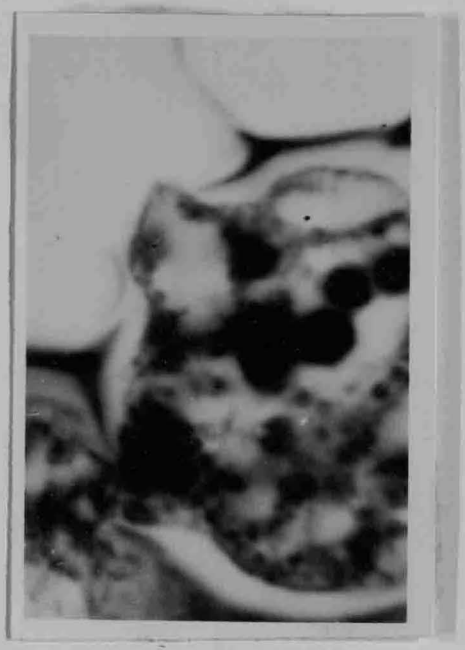


Figure 1

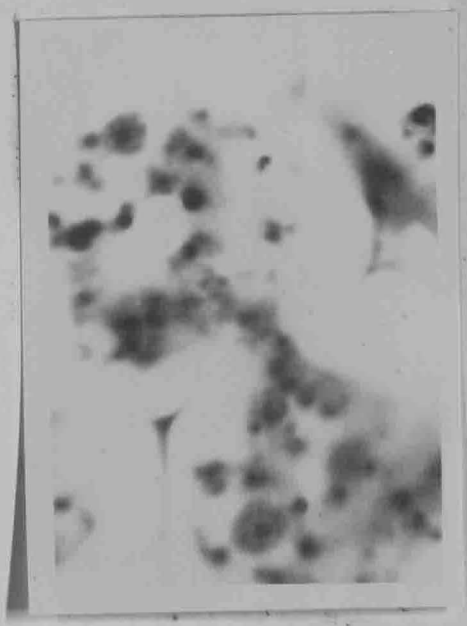


Figure 2

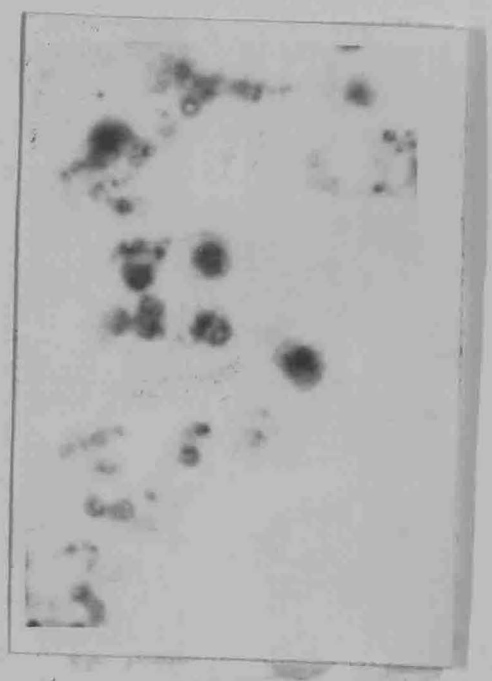


Figure 3

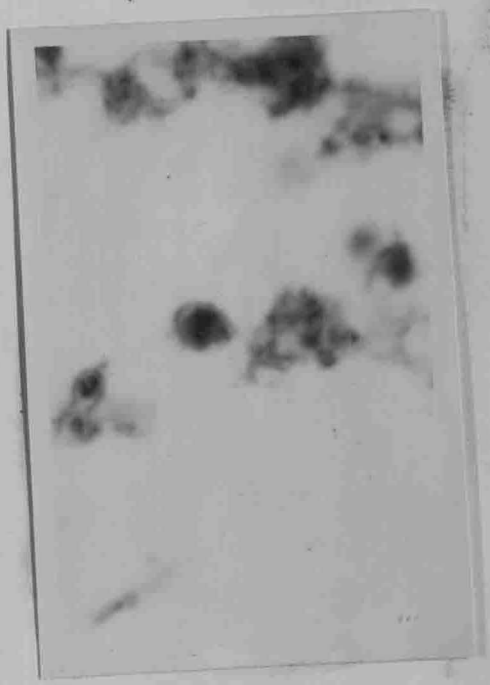


Figure 4

← 10μ →

Plate 12

Explanation of Plate 13:

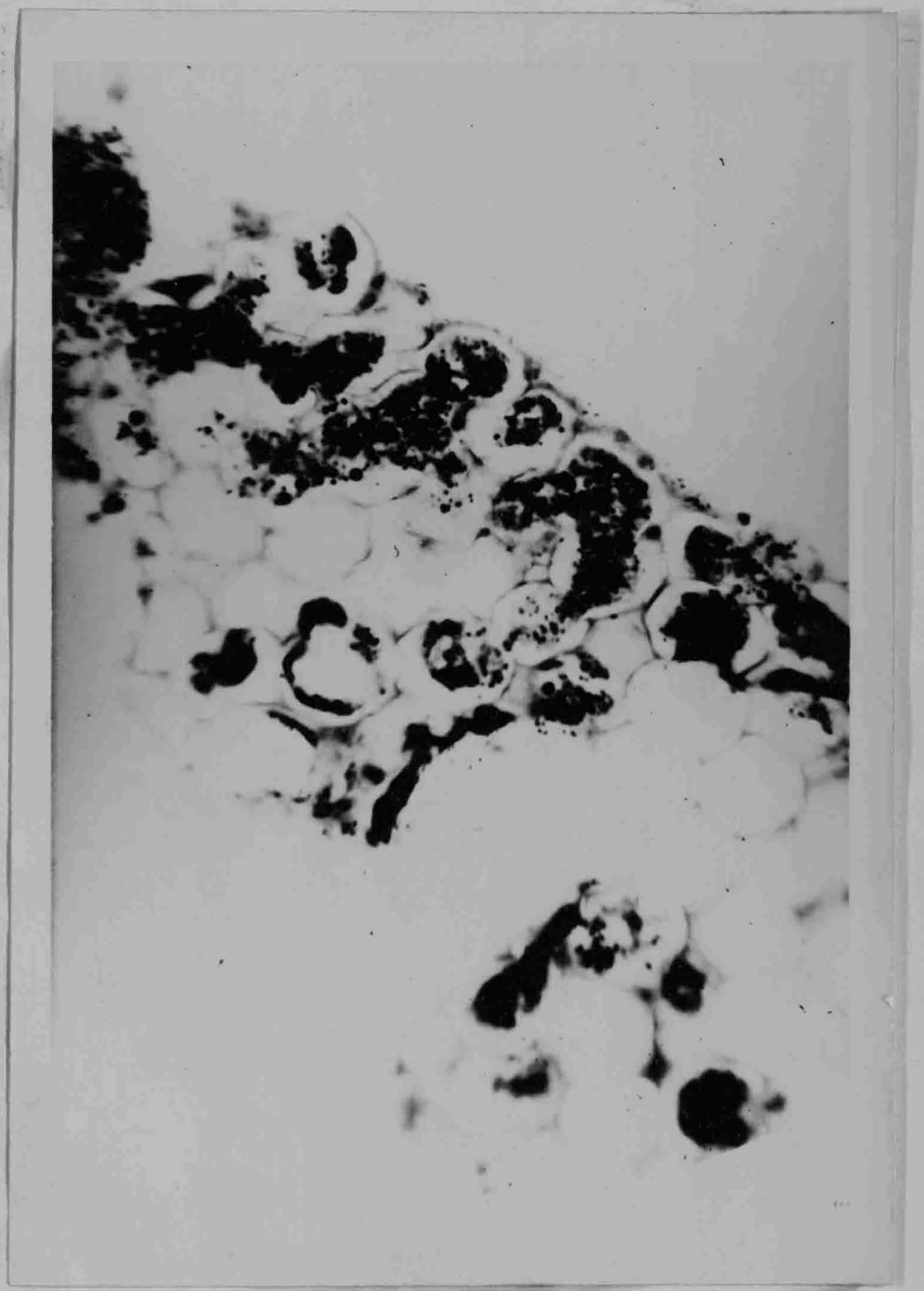
This is all thats left of a once very active mass of germinating spherules. Walls are twisted and torn because of pressure and digestion during reactivation. Two dark concentrations are not identified. The plasmodium is far away, i.e., several centimeters, from this germination center and is feeding.

← 10μ →

Plate 13

Explanation of Plate 14:

Plasmodium is leaving sclerotium. Note that cortex, upper right is still intact for some distance. Spherules germinate and their protoplasts fuse before escape from sclerotium.



←10μ→

Plate 14

Explanation of Plate 15:
(These photographs a re not mentioned in text)

Fig. 1: A single spherule. Photograph is much earlier in the reactivation cycle than Fig. 3, Plate 10. Note six radiating portions from periphery. These are connections with other spherules. Some shrinkage has occurred. This is not a reactivating spherule.

Fig. 2: This is believed to be a dwarf nucleus within a giant amoebule. A positive Feulgen was not obtained for this unsual type nucleus.

Fig. 3: Large spherule in middle of photograph has burst through wall to join smaller protoplast at top left. These three photographs were obtained, using a 10X occular and a 9.7X, N.A. 1.5, oil immersion lens. Magnification was lost, but is approximately that of designation.

Figure 1

Figure 2

Figure 3

Plate 15