On Fertilization in the Saprolegnieae.

BY

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With Plates XXXIV-XXXVI.

TEN years ago, at the suggestion of Prof. Oltmanns of Freiburg, and stimulated by the publication of Humphrey's ('92) admirable monograph on the Saprolegniaceae of the United States, I commenced a study of the cytology of the sexual organs in the genus *Saprolegnia*.

In the years 1894-5, as one of the results of a very long series of observations, the conclusion was reached that fertilization takes place invariably in *Saprolegnia dioica* and at least occasionally in *S. mixta*. The evidence, so far as it concerns the first of these two species, was exceptionally simple, clear, and convincing, but it apparently proved unacceptable to a considerable number of botanists. The evidence rests on three distinct, and in the main, easily verifiable observations, viz. (1) the invariably uninucleate character of the oosphere-origins and oospheres, (2) the invariably binucleate character of the young oospores, and (3) the uninucleate character of the ripe oospores, a feature however which was not so definitely established as was obviously desirable. Special attention should be paid to this statement, for it has been recently suggested by Davis ('03) that my view as to the occurrence of fertilization in the Saprolegnieae rested on other and altogether insufficient grounds, viz. the occasional occurrence of binucleate oospheres (eggs). He says that I have found binucleate eggs (!) in *Saprolegnia* and 'attached much significance to them as evidence of sexuality.' It is unfortunate that Davis uses the term eggs very loosely as synonymous with oospheres and oospores—indeed, as he does not figure the oospore membranes, it becomes difficult to ascertain whether he paid any attention at all to that critical period in development when the oosphere is converted into an oospore. I have seen, in *S. dioica*, on one or two occasions, binucleate oospheres and have of course regarded them as anomalies, even as monstrosities. Certainly, no theories were

based on such isolated observations. Statistically considered they were so rare as to be without significance. The change from oosphere to oospore, even in apandrous forms such as Davis worked with, is easily recognizable and should have been specially noted.

It was, no doubt, a mistake on my part to present the results concerning fertilization in the two species *S. dioica* and *S. mixta*, side by side, as the additional complexity made it difficult for the reader to realize the exact state of affairs without careful study. I ventured, moreover, to suggest that the binucleate oospores observed by Humphrey ('92) in *Achlya americana*, and mistaken by him for oospheres, furnished sufficient evidence for the view that another species of *Saprolegnieae* was also functionally sexual.

There was good ground for this suggestion in the case of Humphrey's work, for he had examined sections and preparations somewhat similar to my own. The endeavour, however, to explain Hartog's ('89, '91) results on the same lines may have been somewhat rash and certainly gave birth to a discussion of a controversial character. The interpretation given of Hartog's observations, however, turns out, so far as it has been tested by new observations, to have been justified.

The view, thus promulgated, that certain species of *Saprolegnieae* were functionally sexual and that the antheridia and fertilization-tubes were not degenerate organs, being obviously incapable of assimilation to that proposed by Hartog, was at once attacked by him. He, however, had no new observations to bring forward in support of his contentions, and as it was obviously impossible to make any real progress in a controversy of this kind, in the absence of fresh evidence, I resolved to continue my work and investigate the genus *Achlya* for myself. It seemed to me at that time, and the progress of research has only served to strengthen the conviction, that the normal occurrence of two nuclei in the young oospores was good presumptive evidence for the occurrence of fertilization. The real value of such evidence depends entirely on the previous demonstration, in my own preparations, of the uninucleate character of the oospheres.

In the year 1897 Miss Dawson collected and cultivated a species of *Achlya*, which, on examination, turned out to be a variety of *Achlya americana*. This form was examined by me in great detail in the years 1897-8, with the result that I ('99) was able to prove that in this species (1) the oospheres are invariably uninucleate, (2) the young oospores are binucleate (in one case out of hundreds three nuclei were observed), and (3) the old oospores are invariably uninucleate. It cannot be too strongly insisted upon that these observations, in the case of this species of *Achlya*, are relatively simple ones, sufficiently so indeed as to form no more than moderately difficult exercises for advanced students. The real difficulties raised by the investigation of the cytological problems of the *Saprolegnieae*

do not arise in connexion with the rigid determination of these relatively simple points. What then was the evidence for the existence of fertilization in the Saprolegnieae in 1898?

In the three species most fully investigated by me, and excluding the admittedly apogamous *S. ferax*, the results were definite enough.

In *Achlya americana cambrica* a practically invariable succession had been proved; the uninucleate oospheres become binucleate oospores, which as they grow older return to the uninucleate condition. This succession, especially so far as concerns the number of the nuclei, may be expressed as a formula:—1, 2, 1.

In *Saprolegnia dioica* the same succession had been shown to occur, but the fusion of the gameto-nuclei being long delayed and difficult to demonstrate, the last stage in the process still required some corroboration. The formula of succession was found to be 1, 2, 1?

In *Saprolegnia mixta* (apparently not the same species as Davis's *S. mixta*) it had been shown that (1) the oospheres were uninucleate, (2) the young oospores either uninucleate or binucleate, and (3) the old oospores invariably uninucleate. The formula of succession was 1, 1 or 2, 1. The formula suggests that parthenogenesis and functional sex exist side by side in this species.

In *Achlya americana*, investigated by Humphrey, it had been shown that the young oospores were binucleate, but there was no evidence as to the number of nuclei in the real oospheres. The old oospores were found to be uninucleate. The formula in this case would be x , 2, 1. The balance of probability is in favour of the view that $x=1$.

It is worth noting that in another species of *Achlya* investigated by Hartog, according to his reiterated statements ('95, '96, '99) the formula would be x , 2, 1 where x is greater than 2. This, if verified, would make the process of fertilization in this case of quite exceptional interest. How far Hartog's views are in accord with the actual facts can only be determined by further investigation. Davis ('03) refuses, as we shall see, to accept Hartog's theory as to the reduction in the number of the nuclei in the oogonium by a process of wholesale nuclear fusions. My observations lead me to conclude that such discrepancies as exist between Hartog's account and those of other botanists must be due either to errors of observation or to such differences in the cytology of the Saprolegnieae as have been shown to exist in the single genus *Albugo* (Cystopus). Further investigations by unbiased observers must be made before the question can now be finally settled. Taking all the evidence into consideration, the view propounded by me in 1899 that 'fertilization is now known to take place in four distinct forms of Saprolegnieae, viz. *Saprolegnia dioica*, *S. mixta*, *Achlya americana*, and *Achlya americana cambrica*,' may be regarded as strictly in accordance with the facts in the case of the three

species examined by me and as very probably correct in the case of the remaining one examined by Humphrey.

To those who are still sceptical as to the occurrence of fertilization in the Saprolegniae two questions may be put. If this remarkable succession concerning the number of nuclei, viz. 1, 2, 1, is not evidence of normal fertilization, what can be the meaning of it? Are botanists prepared to reject all the other very numerous cases of fertilization which are founded on exactly analogous evidence? Most of the proofs concerning fertilization in the Peronosporae—to cite one series of cases only out of many—are of this character. King ('03) concludes that fertilization takes place in *Araiospora* on the basis of the demonstration of the formula 1, 2, 1.

But the strength of the case for the occurrence of fertilization in the Saprolegniae by no means rests on this demonstration of succession in the number of nuclei in the oospheres and oospores. It was proved, at any rate to my own satisfaction, that (1) the increase from one to two nuclei at the point of time when fertilization would naturally take place did not arise by division of the original single nucleus, but, on the contrary, that (2) the second nucleus was first noticeable at the periphery of the oosphere in the immediate vicinity of a fertilization-tube. Indeed, in *Achlya americana cambrica* the tip of the fertilization-tube was traced to a point inside the oosphere (not the oospore) and shown to contain a single nucleus. These additional observations published in 1899 have not met with universal acceptance—indeed they have been subjected to considerable criticism by both Hartog ('99) and Davis ('03).

The criticism, so far as it is relevant to the question of fertilization, is no doubt traceable to three sources:—(1) the incompleteness of the work, (2) the character of the drawings used to illustrate the papers, and (3) the influence of De Bary's views as to the apogamy of the Saprolegniae.

Concerning the first point, nothing need be said. My only concern is that the work may go on towards completion—that progress may be made. With respect to the second, some explanation is perhaps advisable. At the present day it scarcely needs pointing out that every drawing, especially of protoplasmic structures, is of necessity more or less diagrammatic, and that every observer, consciously or unconsciously, decides for himself what conventional forms he shall adopt to convey to others the picture he has himself seen. The convention deliberately adopted by me was to draw every nucleus present in the actual section figured, and to take one optical section, out of six or seven possible ones, as a rough guide for filling in the cytoplasm. In the endeavour to be as realistic as possible the nuclei in the original drawings were not drawn boldly enough. The fine shades of difference in form and colour, perceptible readily enough under the microscope to the trained eye, when realistically copied in black and white, did not show sufficient contrast. The lithographer, with the original draw-

ings before him, had a difficulty in interpreting the nuclear figures correctly, and readers of the paper have, at any rate in some cases, been led completely astray. Davis ('03) and Hartog ('99) have even gone so far as to find in these imperfect conventional figures—separated, that is to say, by two removes from the actual facts—structures which the author of the paper was not able to see in the original preparations. An expert observer is always able to see more than he can reproduce in a figure, and every piece of original work must, therefore, be finally judged by the verdict of his fellow workers as based on collateral or confirmatory original observations. If special reference to the illustrations which have been used as the vehicle to communicate the result to others is resorted to, it must be done with due caution. If the author has been simply unfortunate in his methods of presentation, the verdict in his favour may be delayed, but it will not be permanently withheld. But there is a real value in the criticism as to the want of detail in the drawings, in so far as that depends on the imperfection of the preparations themselves. The material used in both researches had been fixed by means of mercuric chloride. I am convinced that, so far as concerns the finer details of the structure of the nucleus, this method of fixation is quite unsatisfactory. Nevertheless the better methods used by me recently, and to be described hereafter, have not led to results in any sense contradictory of the earlier ones. The new results, indeed, confirm the old in every essential particular. Concerning De Bary's influence much might be said if it were not already fairly well known. It is interesting, however, to contrast his mental attitude with that of Davis. De Bary ('87) says, referring to Pringsheim's work:—'If there is really an open communication in these species between the antheridium and oosphere . . . we must admit a fertilization in their case.' Davis ('03) says, at p. 246:—'The writer cannot better sum up his attitude towards Trow's opinions on sexuality in the *Saprolegniales* than by defining them as *not proven* and *improbable* in the face of the mass of observations upon which botanists have generally agreed that the group is apogamous,' and again, at p. 236:—'The writer cannot justify Trow's conclusions in this matter, believing them to be premature as to evidence and illogical as to probabilities.' One is tempted to ask what is meant by premature evidence and illogical probabilities. Further comment is needless. The greatest student of the *Saprolegniae*, had he been able to pronounce judgement on the same facts, would, I imagine, have expressed himself differently and somewhat as follows:—If this remarkable succession in the number of the nuclei be confirmed, and there be no question of a nuclear division, we must at length admit that fertilization takes place in three species of *Saprolegniae*—the view concerning the species destitute of antheridia, however, of course, remains unaltered.

It must be admitted that the most difficult problem in the cytology of

the sexual organs, that of the fate of the supernumerary nuclei in the oogonium, had not been very successfully attacked during the course of these investigations. My view that the supernumerary nuclei disappeared by a process of digestion and absorption was distinctly opposed to that held by both Hartog and Humphrey.

The difficulty of following the process of degeneration of the nuclei in detail was so great that I determined to study a member of the Peronosporae with a view to getting some practice in following the degeneration of indubitable superfluous nuclei. With this end in view a special study of a species of *Pythium* was made in the years 1899 and 1900. It was very gratifying to find that with the same methods as had been employed in the case of the Saprolegnieae, practically the same results were obtained; in particular it was easy to trace exactly the same succession as regards the number of nuclei in the reproductive organs. Moreover, it became apparent that chromacetic acid was an excellent fixing reagent for use in this group of plants.

With the knowledge gained by these investigations a fresh start was made in the study of the Saprolegnieae in 1902. The first suitable species isolated, which was collected for me by one of my pupils, Mr. Pole-Evans, proved to be *Achlya polyandra*, Hildebrand. I had already spent twelve months upon the investigation of this and practically completed my work when Davis ('03) published his interesting observations on the cytology of the oogonia in an apandrous species of *Saprolegnia*. This well-known cytologist fully confirms the observations already made by me in that genus, and delineates with characteristic care and diagrammatic clearness the details of nuclear structure. The great merit of the research from the cytological point of view rests on the discovery of a structure which the author classes as a 'coenocentrum,' but which has all the appearances of a centrosome and its astrosphere. Although this structure has not, in my opinion, the significance which Davis attaches to it, yet it may be conceded that its presence materially helps the observer to clear up the difficulties inherent to the study of the nuclei in these sexual organs. Unfortunately, its discoverer made comparatively little use of his opportunity, although he seems to have been fully aware of it. Davis's paper is mainly occupied with theoretical discussions of a highly controversial character. The author rejects Hartog's observations as to wholesale fusions of nuclei, and adopts the theory first promulgated by me, that the supernumerary nuclei are digested and absorbed by the ooplasm. As already noted above, however, he refuses, with very marked emphasis, to accept my observations and conclusions as to fertilization, although, strangely enough, they are, so far as the observations are concerned, perfectly consistent with his own. His reason for this somewhat singular attitude of mind appears to be

based on the fact that he attaches extreme importance to the occasional occurrence of binucleate and trinucleate oospores in the apandrous species examined by him, a phenomenon which appears from its infrequency and the author's drawings and methods of culture to be in all probability of a pathological character. It is simply unfortunate that Davis should have ventured to enter upon such a controversy upon the basis of the examination of an obviously apogamous form; especially as it is clear that his methods would, if diligently followed out on appropriate material, have inevitably led him to the perception of the truth as to fertilization. There can, indeed, be no doubt at all that if he had examined a typical species—one i. e. with antheridia—he would have observed the succession in the number of the nuclei which has been already described, and have been compelled to modify his views accordingly. While his material was admirably suited to enable him to study the development of the oospheres and oospores, untrammelled by the difficulties associated with the presence of antheridia and fertilization-tubes, it is almost inconceivable that any one should choose it to enable him to take part in the settlement of those controversial questions which have been raised by the recent study of species of *Achlya* and *Saprolegnia*. Such an apogamous form as was examined by Davis is exceptional in the genus *Saprolegnia*. I have not yet succeeded in my endeavours to collect one in the genus *Achlya*. In cytology, as elsewhere, it is best and safest to proceed from the rule to the exception and not from the exception to the rule. These observations of Davis, however, impelled me to seek for another species of Saprolegnieae in the hope of verifying his observations on the so-called 'coenocentrum.'

With the assistance of Mr. Pole-Evans, *Achlya De Baryana*, Humphrey, was obtained, and proved in every way a most desirable subject for experiment and observation. The communications to be made on these two species of *Achlya* will, it is to be hoped, dispose finally of the vexed question as to the occurrence of fertilization in the Saprolegnieae.

METHODS.

It is very desirable that the work already done on the Saprolegnieae should be extended and amplified as well as confirmed. A short account of the methods used by me, the result of the experience of the last ten years, may be of service to those who wish to specialize in this promising field of research. It has been pointed out in earlier papers that the Saprolegnieae may be easily collected and cultivated. Fine cultures of single well-identified species are highly desirable for cytological work, and they are readily obtainable if attention be paid to a few simple particulars. Excellent nutrient material for the cultures is provided by house-flies, which should be killed by chloroform, placed in plugged test-tubes and thoroughly sterilized. These flies will, if properly prepared, keep in good condition for several

years—a point of no small importance. The best and most convenient culture vessels are Petri dishes, and these too should be sterilized. Sterilized tap water is suitable where the water is sufficiently free from lime. If lime is precipitated on sterilization, distilled water or rain water should be used. On no account should cultures be attempted on white of egg, chopped beef, or other similar substrata, unless the inoculating material has been entirely freed from bacteria and other micro-organisms. The flies, having been transferred to Petri dishes half filled with water, are inoculated by cuttings of hyphae from healthy cultures, a single filament being transferred by means of a sterilized needle. When this process is repeated with the requisite care for a few generations, micro-organisms are practically eliminated, and if an even temperature be maintained the sexual organs appear on the cultures in great abundance with clock-work regularity a few days (generally about five) after inoculation. I have kept such cultures going for twenty to thirty generations in perfect health and vigour, and such as these were alone used by me for fixing. The first essential in cytological work is thoroughly healthy and vigorous material. The time and labour spent in the preliminary study of the living plant meets with its due reward in the greater constancy and accuracy of the final results. All sorts of anomalies occur when the conditions of life are unfavourable, as the *Saprolegnieae* are exceptionally susceptible to changes in their environment. Both the species of *Achlya* collected for me by Mr. Pole-Evans were found in the hard waters which everywhere flow from the lias in the Vale of Glamorgan. They frequently occur in a curious stunted, coral-like form which is almost, if not quite, sterile. Such cultures, if transferred to distilled water, develop normal growths at once.

The best fixative is a solution of chromic and acetic acids in water in such proportions that the percentages of the two acids are respectively 0.7 and 0.3. The much weaker solution used by Davis was tried, but the results in *A. De Baryana* were distinctly bad. The solution should be allowed to act for twenty-four hours and the material should then be washed in running water for another twenty-four hours. The transference to absolute alcohol is effected very readily and safely by the use of the diffusion shells of Schleicher and Schüll. All danger of shrinkage is avoided by this method and it has the merits of being easy to use and certain in its effect. The transition from absolute alcohol to paraffin may be successfully made by means of xylol, chloroform, or cedar-wood oil, but care must be taken to avoid sudden transitions at every stage of the process. The material itself should never be handled either with lifters, forceps, or other tools, at any time, but rather floated gently from one vessel to another when changes are necessary. The two essentials for success are undoubtedly fine healthy cultures free from micro-organisms and an appropriate fixative. The method of applying the fixative is important. Two cultures grown

side by side in the same Petri dish, apparently exactly alike, fixed simultaneously with chromacetic acid of the usual strength, and subsequently treated alike in every respect, yielded different results. One yielded me my finest preparations, the other provided only moderately good material.

Sections 7.5μ thick are sufficiently thin to show nearly all the details perfectly, but I have relied entirely on sections 5μ thick for the work described below. Sections which are thinner than this are more difficult to work with and there is as a rule no special advantage to be gained by their use.

Most of the nuclear stains give good results, but gentian-violet is undoubtedly the best for general work. This was used, according to Gram's method, with or without a second stain such as eosin. Very fine preparations were obtained with both gentian-violet and fuchsin when differentiation was carried out by chromic acid or picric acid solutions. Flemming's triple stain proved to be an excellent one for most purposes, but was less constant in its action than the others. Haematoxylin stains are the least suitable, for the microsomata in the protoplasm—the vibrioid organoids of Swingle ('98)—take the stain so greedily as to unduly mask the nuclear structures. With sections 1μ thick, however, this stain would be the most satisfactory of all. The demonstration of critical and difficult points of structure may be carried out with any one of these stains, provided the material has been properly selected and prepared.

NOTE ON NOMENCLATURE.

The synonymy of the two species of *Achlya* with which we are concerned is somewhat confusing, both species having received the same name, *Achlya polyandra*. Humphrey ('92) recognized the confusion when preparing his monograph on the group and cleared up the difficulty satisfactorily. Fischer's ('92) view is in practical agreement with that of Humphrey, although he left the synonymy in its original confusion. I have only to add that I accept the conclusions of the American monographer. It will suffice to point out here that the first species examined by me should be named *Achlya polyandra*, Hildebrand; it is *Achlya gracilipes*, De Bary. The second species should be named *Achlya De Baryana*, Humphrey; it is *Achlya polyandra*, De Bary.

OBSERVATIONS ON *ACHLYA POLYANDRA*, HILDEBRAND.

The examination of this species occupied about eighteen months and many interesting facts came to light in addition to those with which we are at present concerned. I shall restrict myself to indicating the evidence procured as to the occurrence of fertilization, and such cytological phenomena as are associated therewith. Observations on the living

material revealed no new facts of interest. The examination of sections showed almost at once that the cytology of this species of *Achlya* corresponded closely with that of other species of *Saprolegniae* which possess perfect sexual organs.

There is no necessity for a detailed description of the observations. The essential points can readily be appreciated by reference to Figs. 1 to 13. The oogonia and antheridia are multinucleate (Figs. 1, 2, and 3). The nuclei, although already much more numerous than is necessary to meet all the requirements of the sexual process, undergo an indirect division in both oogonia and antheridia (Figs. 1, 2, and 3). The oogonial nuclei are reduced in number by the degeneration and absorption of the excess before the oospheres are fully formed, and each oosphere as it rounds itself off is provided with a single, centrally-placed nucleus (Figs. 4, 5, and 6). The fertilization-tubes grow into the cavity of the oogonium, and when they reach the oospheres, each of these acquires a cell wall and a second nucleus (Fig. 7). The oospheres become, in fact, young oospores. That the entry of the second nucleus could not be traced seems to be of relatively small importance so far as the demonstration of the occurrence of fertilization is concerned. The two nuclei of the young oospore approach each other, but the actual fusion is delayed until the oospore wall has acquired a considerable thickness (Figs. 8, 9, and 10). Ultimately the nuclei invariably fuse, as is proved by the fact that the old oospores are always uninucleate. The superfluous nuclei and protoplasm of the antheridia undergo very slow degeneration and eventually disappear. In the case of this species no single exception as to the number of nuclei has been discovered;—the formula of succession, based on the examination of hundreds of normally developed sexual organs, is 1, 2, 1, and absolutely invariable.

Several points in the cytology, however, deserve special attention. In the anaphases of some of the nuclei in the case of a series of sections through one oogonium, appearances were noted such as are represented in Fig. 1, *a* and *b*, which suggested that a second mitosis sometimes took place immediately after the first without the normal intervening resting stage. I spent much time and labour in following up this clue, but failed to secure any better results in this species. It was partly this fact that impelled me to seek an additional species for further investigation.

The degeneration of the nuclei during the formation of the oospheres was also very difficult to follow; the experience gained in the study of degenerate nuclei in *Pythium* was of very little assistance. It was certainly not difficult to distinguish a single, central, deeply stained nuclear structure in each 'origin' and oosphere, but after Davis had published his paper I was obliged to admit that there would be ample justification for the criticism that in this case the 'coenocentrum,' if present, would be grouped

with its accompanying nucleus and be regarded as one body. In fact, it is extremely likely that during imperfect fixation of the nuclei and 'coenocentrum' a fusion of these very distinct bodies actually takes place—a result, however, of no special significance so far as concerns the demonstration of the occurrence of fertilization. The nuclear membrane of the centrally placed nucleus of the 'origin' is very difficult to demonstrate. I frequently could not satisfy myself as to its presence at this stage. It is probable that the nuclear membranes of the nuclei of the oospheres make their appearance just at this point.

The representation of a fertilization-tube in direct, open communication with a young oospore in Fig. 11 is noteworthy. It might well be accepted as a positive proof of fertilization. It is, however, an anomaly; the tubular neck has a relatively thick wall, a feature invariably absent from normal fertilization-tubes; and the adjacent section, represented in Fig. 12, shows a fertilization-tube already attached to the oogonium and delimited from it in the usual manner by a definite transverse membrane. There can be little doubt that the normal fertilization was effected by the second of these tubes and that the first one, notwithstanding its clearness, is anomalous. It is the only one of this type seen, and yet hundreds, if not thousands, of fertilization-tubes have been examined in normal contact with the young oospores. Such an anomaly, though obviously pathological, or at any rate very exceptional, is however not without its significance. Special reference should be made to Fig. 13, showing a typical fertilization-tube in direct communication with the oosphere or oospore. In this case the staining was so dense that the nuclei could not be seen at all, but an area of protoplasm in the oosphere, just opposite to the fertilization-tube, was stained in exactly the same way as the protoplasm of the tube itself, and the direct continuity of the protoplasm of the two organs was, without difficulty, established. This preparation, though useless for the study of karyology, sufficed to settle the question as to the existence of an open communication between the fertilization-tube and the oosphere, and furnishes some evidence for the view that protoplasm passes over from the fertilization-tube to the oosphere. It is interesting to note the way in which the gameto-nuclei approach each other, as shown in Fig. 8. The nuclei are oval and possess deeply stained granules at the somewhat pointed anterior ends. These granules lie apparently inside the nuclear membrane. They are typical, not exceptional, structures in this species, at this stage of development—at any rate with the methods of preparation employed by me—and are probably to be connected with the existence of centrosome-like structures which have fused more or less completely with the nucleus. However, the newer observations made on the second species—the details of nuclear structure being worked out more thoroughly in that case—will throw additional light on this phenomenon.

It is clear that the work on *Achlya polyandra*, Hildebrand, suffices to enable us to add another species to the list of the Saprolegnieae in which functional sex has now been established.

OBSERVATIONS ON *ACHLYA DE BARYANA*, HUMPHREY.

Figs. 14 to 34 serve to illustrate all the fundamental features of the cytology of this species. These may be compared with the corresponding figures published by me in '95 and '99, as well as with Figs. 1 to 13. Novel or controversial points will alone be discussed in this paper.

The structure of the nucleus in the Saprolegnieae and the first mitosis in the oogonium. The resting nucleus of the Saprolegnieae has, in common with many other fungal nuclei, been frequently figured as consisting of a central chromatic body separated from the investing nuclear membrane by an achromatic perfectly hyaline substance. Davis ('03) occasionally represents the nucleus as of this type, as e. g. in Figs. 31 to 35 of his recent paper. However, Davis's description, with which most of his figures agree, gives a correct account of the actual appearances. He says, 'There is a nuclear membrane enclosing a well-differentiated nucleolus, prominent by its size and staining qualities. Much less conspicuous, but readily demonstrated in well-fixed material, is a loose linin network which contains the chromatic material.' The observations summed up in this statement agree with my own recent ones; they do not differ, indeed, in point of fact from those I published in '99. Still, I must object to the view that the central body has been proved to be a nucleolus. This is not so much a question of the relative value of preparations, concerning which every botanist may be allowed to have his own opinions, but rather of the interpretation of observations. If this conspicuous structure is a nucleolus, it is a giant of its kind, and such nucleoli in the higher plants would excite considerable interest. I have looked in vain for figures of such. My present conception of this problematic body, which I provisionally regarded as a 'chromosome' in '95, may be best realized if one imagines the nucleolar matter in the nucleus of a lily to increase in amount to such an extent as to half fill the nuclear cavity, and in doing so, to enclose, not displace, a large portion of the pre-existing linin network and the associated chromatin. This view, first promulgated in '99, I am not yet prepared to discard. It is based mainly on observations made on the central body during the pro phases of karyokinesis. It is in accordance both with Davis's figures and the descriptions of his observations, but is in conflict with his interpretation of these; whether in the light of the recent researches of Wisselingh ('00) this view finds corroboration or not, seems to me of little importance. According to Wisselingh, the so-called nucleoli of *Spirogyra* give rise during karyokinesis to distinct chromosomes. The nuclei of the Saprolegnieae are not very suitable objects for the elucidation

of this rather difficult, and in the present connexion somewhat irrelevant, problem. A comparative critical study of nucleoli is obviously a desideratum. The recent work of Wager ('04) and Williams ('04) make it clear that the current conceptions of the structure of the nucleus and of its behaviour during mitosis will have to be modified in several respects.

My observations, so far as they concern other features of the karyology, agree with those of Davis in the following features:—(1) the intranuclear character of the spindle, (2) the persistence of the nucleolus (as a much smaller body than the original central mass) up to the metaphase stage in mitosis, and (3) the absence of centrosomes and astrospheres. It is clear to me, however, that the number of chromosomes in the nuclei of the species is certainly greater than four and probably eight, and that the nuclear figures are not nearly so regular as those figured by Davis. Moreover, the divisions of the nuclei are not so exactly synchronous as in the cases figured by him. The arrangement of the daughter-chromosomes as in Fig. 16 *a* suggests that a second mitosis is to take place, at any rate in some cases. The conditions seem to be more complex in this species than in Davis's apandrous *S. mixta*.

Whilst the first mitosis is in progress the layer of protoplasm lining the oogonium gets thinner and thinner and it attains its minimum thickness before the second mitosis is initiated. It is at this stage that the real cytological difficulties have to be faced. It is noteworthy that up to this point in the development of the oogonia, *Achlya De Baryana* does not differ in any fundamental feature of its cytology from the apandrous species investigated by Davis. Such slight differences as are noticeable are probably traceable either to specific variations in the plants themselves or to differences in the methods employed in their examination.

The second mitosis. Davis recognized the difficulties which naturally arise at this stage, but does not appear to have succeeded in solving the main problem. He seems to have overlooked the fact that the stage in the development of the oogonium immediately preceding that of 'balling' is readily distinguished in sections by the thinness of the parietal layer of protoplasm. His figures appear to be founded on the examination of tangential sections alone. Making all allowance for possible errors of interpretation, I am unable to reconcile his views with the observations made by me in *Achlya De Baryana*. Future investigations must decide to what extent these differences are founded on errors of observation and judgement, or on differences of fact.

When the anaphases of the first mitosis are over a single row of nuclei may be observed in the thin parietal layer of protoplasm (Fig. 17). The nuclei are not so numerous as they ought to be, and as some of them—marked *k*, *l*, *m*—stain so badly as to leave little doubt that they are undergoing dissolution we may infer that the absorption of the superfluous

nuclei has already commenced. The most distinct and deeply stained nuclei are in the spirem stage (Fig. 17 *a, b*.) Later prophases are to be seen (Fig. 17 *c*). Some of the nuclei, e.g. those marked *e, f, g, h*, are provided with deeply stained granules attached to the outside of the nuclear membrane. In one case, marked *j*, three granules were seen in this position, with protoplasmic threads radiating outwards from them. Such an assemblage of nuclei is of course very puzzling, and as this and the following stages are difficult to fix properly, there is room for errors in interpretation. Contrary to Davis's experience, the staining is not exceptionally difficult, the detail comes out perfectly with all the methods employed. The most natural inference to be drawn from these observations seems to be, that a certain number of nuclei are selected to undergo a second mitosis, and that the remainder are doomed to degeneration.

The further study of the fate of these dividing nuclei is very difficult, and, but for the successful fixation of one culture, might have proved barren of real result. One oogonium, thoroughly examined in a series of seventeen sections—a week's work—furnished examples of the metaphases and anaphases of this second mitosis. Some of these sections are represented, wholly or in part, in Fig. 18. It will be observed that the dividing nuclei are associated with small resting nuclei which still retain their normal characters and with degenerate nuclei of various types. The nuclei do not divide synchronously. In the late metaphases, as shown at *a, b, c, d*, a small elongated spindle is seen. It is noteworthy that this appears to consist always of three threads. Whether the two outer lines represent spindle threads or the optical sections of the nuclear membrane may be a matter of controversy, but an examination of the nuclear figure at *a* leads to the belief that the nuclear membrane is absent and that the chromosomes pass polewards along all these threads in a less regular manner than is the case during the first mitosis. This division, indeed, resembles in many of its features the corresponding division in *Albugo* described by Stevens ('99) and Ruhland ('03). At the poles of the spindles there can generally be detected a small deeply stained granule, which, as it is the centre of a system of polar radiations, must be regarded as a centrosome, the radiating threads constituting its astrosphere or aster. The rays of the astrosphere can be traced without any break of continuity right up to the centrosome. Late anaphases are seen in *e, f, g, h, j*. The two nuclei at *e* are daughter-nuclei, the connecting threads of the spindle being no longer continuous. One of the daughter-nuclei has turned through an angle of nearly 90°. The new nuclear membrane makes its appearance at this point, but is at first not readily demonstrable. At *f, g*, the centrosomes somewhat obscure their nuclei. These, although demonstrable, have been omitted from the drawings for the sake of clearness. The nucleus figured at *g* had neither centro-

some nor astrosphere. Cases such as *g* and *h* require attention. In *h* the association of the centrosome and astrosphere with its nucleus is clear. The centrosome is in close contact with the nuclear membrane. From a study of *g* an observer might conclude that the centrosome was attracting to it the adjacent nucleus. Considerations such as these, combined perhaps with preconceived ideas as to the distribution and physiological significance of 'coenocentra,' may have led Davis astray when working at this very complex problem.

Against the adequacy of the proof furnished here of a second mitosis taking place, it may be urged that the peculiar appearance of elongated spindles may be explained more easily and naturally on the assumption that we have before us a case of the division of the centrosome and the formation of an extra-nuclear spindle, the nucleus itself remaining undivided or its division being overlooked. Such a contention must not be lightly dismissed. Certain observations, relatively few in number serve, however, to dispose of it effectually. Nuclei in the nuclear-plate stage are occasionally seen as at *k* and *l*—the latter, it is well to note, in an antheridium. These nuclei are provided with a nuclear membrane, and are certainly undergoing a second mitosis. A comparison of the earlier stage in the development of the oogonium represented in Fig. 17, where all the nuclei have completed the first division, and of the still earlier stages represented in Figs. 15 and 16, where all the nuclei are undergoing division, with this one, must convince any unprejudiced observer that there can be no question here of the inadmissibility of the explanation that these early metaphases may belong to a deferred first mitosis. The dissimilar elongated nuclei seen in late metaphases and anaphases are, no doubt, derived from the earlier metaphases by the loss of the nuclear membrane and the elongation of the spindle. Harper ('99) has figured a similar elongation of the spindle in the nuclear divisions in the ascus of *Lachnea*. The apparent absence of the astrosphere in early metaphase, when at best it must be very feebly developed, is of little importance. It is only in very late anaphases that it makes itself really conspicuous.

Degeneration of the supernumerary nuclei. There are one or two points which must be clearly borne in mind when endeavouring to clear up the difficulties associated with the actual observation of the degeneration of the nuclei. A functional nucleus is only definitely recognizable by its structure, and in general that structure is only demonstrable in well-stained preparations. Degeneration is associated with loss of structure. Degenerate nuclei must therefore soon become unrecognizable. The degeneration and digestion of the nuclei is very rapid in *Achlya*, indeed much more so than in *Pythium*. Badly-stained nuclei associated in the same section with well-stained ones must be regarded as degenerate. We have to try and arrange these in a consecutive series to form some idea of the process of

degeneration. At one end of such a series the nuclei would with difficulty be recognized as degenerate at all—at the other end it would in most cases be impossible to feel sure that they really were nuclei. Notwithstanding much study it has proved impossible to give any definite account of the various stages in degeneration which the nuclei pass through. Degeneration may apparently commence either when the nuclei are in the resting condition, or when in the prophases or even the metaphases of mitosis. The nuclear membrane seems to disappear, the surrounding protoplasm to suffer a change which causes it to take up stains more readily, and the central mass—the doubtful nucleolus—to break up into smaller portions or remain intact, according to circumstances (Fig. 18, *m, n, o, p*). A blurred astrosphere-like arrangement of rather thick but indefinite protoplasmic filaments is sometimes associated with these degenerate nuclei, as seen in Fig. 18, *q, r, s*. I would suggest as a provisional explanation of this rather curious feature that they are the daughter-nuclei of the second mitosis undergoing degeneration along with their associated astrospheres. This observation is difficult to reconcile with Davis's contention that his 'coenocentra' select and nourish the functional nuclei.

In the last phases of degeneration, after the supernumerary nuclei have entirely vanished as such, large deeply-stained granules are to be found in the ooplasm, as shown in Fig. 19. These may best be regarded as certain by-products of the degeneration process. They correspond somewhat to the similar structures figured by Davis, but are much fewer and larger.

Development of the astrospheres and ovocentra. Before all the superfluous nuclei are quite destroyed, the process of the formation of the oospheres (balling) begins. The astrospheres grow rapidly in size. The protoplasm associated with them loses its larger vacuoles and becomes fine grained. The nucleus remains in close contact with the centrosome—Fig. 19, *a*—and its chromatin seems to concentrate itself in the immediate neighbourhood of that body. As Davis has already pointed out, it would be very easy to mistake this dual structure for a single one, even in fine preparations. The centrosome absorbs nuclear stains with the same facility as the chromatin of the nucleus. In good preparations the centrosome, like the nucleus itself, is readily demonstrable by its structure. It always appears, when well developed, as a single granule, from which there radiates outwards in all directions a relatively small number of protoplasmic filaments. These filaments are traceable right up to the centrosome and are continuous with it. Centrosome and astrosphere correspond very closely with those in *Pellia*, figured and described by Farmer and Reeves ('94). The nucleus can be recognized with certainty even when it lies immediately under the centrosome. Nuclei occupying this position have not been inserted in the drawings for obvious reasons. The nuclei are not shown consequently in Fig. 19, *b, c, d*. In one case—Fig. 19, *d*—I

observed a well-developed nucleus, its centrosome and astrosphere, in close association with a recognizable nucleus bearing at one end a few astral rays. Some botanists might be prepared to argue from this isolated occurrence that a fusion of nuclei takes place at the commencement of 'balling.' The more reasonable explanation is, I think, that this solitary supernumerary nucleus was the last recognizable survivor of the superfluous nuclei, and destined to disappear by degeneration in due course, like its fellows. In fact, it would probably have been overlooked had it not had associated with it a few astral rays. In the same section two small indefinite astrospheres with the nuclei no longer definitely demonstrable were noted, viz. at *e* and *f*. As 'balling' proceeds the astrosphere continues to increase in size until it is a very conspicuous structure indeed. It reaches its maximum development just before the separation of the 'origins' in the process of the formation of the oospheres (Figs. 20 and 21). The rays of the astrosphere, it will be noted, are mainly directed towards the oogonial walls in radial sections. The nucleus has a well-defined nuclear membrane, and the chromatin is aggregated at the end next the centrosome, forming there, as it were, a deposit on the inside of the nuclear membrane. The nucleus thus acquires at this stage a distinct vesicular character—one specially remarkable from the absence of the usual central body. *At this stage the 'origins' are invariably uninucleate.* The absolute determination of this point is necessary in order to prove fertilization by means of the order of succession in the number of nuclei in the oospheres and oospores. Eleven oogonia were examined critically. These contained respectively 5, 13, 11, 10, 8, 8, 9, 11, 4, 6, and 14 'origins.' Ninety-nine origins were thus examined. All were uninucleate. Many other oogonia were casually examined in the course of the investigation, and in no case were two nuclei observed in one 'origin.' At about this time the fine-grained protoplasm surrounding the nuclei in the centre of the 'origins' begins to stain more deeply and thus to acquire a certain appearance of individuality. I propose to call this central mass of material, for convenience in description among other reasons, the *ovocentrum*. I do not regard this ovocentrum as a morphological unit in any sense, but rather as a transitory appearance depending primarily upon the forces acting in the oosphere. The materials—whether living protoplasm or reserve food products—by virtue of which the acting forces put themselves in evidence, play apparently a passive part; to use a hackneyed simile, they behave as iron filings do to a magnet. It is to this body that I have hitherto applied in other species of plants the term 'coenocentrum.' It is clear that the term 'coenocentrum,' first proposed by Stevens ('99), and used afterwards by Davis ('00, '03), Ruhland ('03), myself, and others, has been applied to more than one type of object. The use of the term 'coenocentrum' has no doubt facilitated the study of the sexual organs of the Phycomycetes, but it can scarcely be regarded as

a happy one, for the structure in question is actually at the centre of an ovum. The term in question is not unsuitable for the case in which it was first used by Stevens—the oospheres of *Albugo*—for the numerous nuclei in these can be considered as having a common centre. Ovocentrum appears to be a more suitable term for general use, and will be used by me to denote the aggregation of granular protoplasm about the astrosphere and nucleus in the oospheres of this *Achlya* and the other members of the Phycomycetes investigated by me. Ruhland criticizes my comparison of such an ovocentrum to a 'whirlpool in a river.' Now, as the simile was used to call attention to the view that the constancy of form was maintained rather by a constant force than by a constant substance, there seems no real difference of opinion between us except as to choice of illustration. Surely, though, a whirlpool whose relatively permanent form depends primarily on the force of the rushing water, and whose actual particles, whether of swirling water or floating foam, are continually entering and leaving it, furnishes a not inappropriate simile. When the ovocentrum takes the form of a single well-defined granule or globule, with a definite limit, perhaps even a limiting membrane, as figured by Davis ('00) and Stevens ('99) for *Albugo*, the simile is no doubt an inadequate one. The fact, however, remains, that even here we are dealing primarily with force rather than with substance—the acting force is regular and necessary, the substance is more or less irregular and accidental. Figs. 22, 24, 25, 26, 28, 29, and 34 lead one to infer that the ovocentrum is the result of forces emanating from the region of the centrosome. The question of the function of the centrosomes themselves has been the cause of much speculation, but it is one that can scarcely be attacked profitably in this connexion. We require more detailed observations on their origin and distribution in plants. My observations, indeed, lead me to doubt whether the centrosomes and astrospheres are themselves anything more than manifestations of the forces acting at certain points in the oospheres, or better, at points in the immediate vicinity of the nuclei. To build up theories of chemiotaxis on such slender foundations as the juxtaposition of centrosome and nucleus, as has been done by Davis, appears to me to lead to no result of real value.

The entry of the sperm nucleus into the egg. Fertilization. Three oogonia, containing respectively 4, 17, and 11 oospheres, were critically examined. Each oosphere had the structure represented in Fig. 22. It was provided with a single nucleus in the resting condition, a centrosome and astrosphere, and an ovocentrum of dense protoplasm. Many other oospheres were casually examined, and if fertilization-tubes had not reached them, they always proved to be uninucleate, and to possess the structure shown in Fig. 22. The accurate demonstration of the entry of the sperm-nucleus into the egg is excessively difficult, and has only been satisfactorily carried out on one culture fixed in chromacetic acid. The fertilization-tubes, as

in all the other sexual species, can be traced up to the oospheres. With these they come into close contact—one which, once established, persists for a considerable time, and can be recognized even in fairly old oospores. It was the nature of this contact (see Figs. 26, 28, and 29), combined with the variation in the number of the nuclei, that led me, years ago, to look carefully for proofs of the entry of a sperm-nucleus. The fertilization-tube, after coming into close contact with one oosphere, generally sends out a lateral branch which may, and frequently does, come into contact with another oosphere. This fact, together with the failure to note the constant adhesion of the fertilization-tubes to the young oospore membranes, apparently led Hartog to regard the fertilization-tubes as functionless organs. A study of Figs. 23, 24, and 25 will suffice to show that the apex of the fertilization-tube not only indents the oosphere, but really penetrates into the ooplasm, and by virtue of the solution of its membrane allows a direct protoplasmic continuity to be set up between the fertilization-tube and the oosphere. The passage of a nucleus from the one organ to the other may be traced (Figs. 23, 24, 25, and 26). An admirable proof of the continuity of the protoplasm in both organs is furnished by Fig. 23, which may be interpreted as meaning that the nucleus is accompanied by an appreciable quantity of protoplasm—a gonoplasm is indeed demonstrated. Such a figure reminds one very much of the nuclei squeezing themselves through the sterigmata of the Basidiomycetes, as figured by Ruhland ('01) in *Hypholoma*. Attention may be directed to one or two other points of interest. The protoplasm in the oosphere in the neighbourhood of the fertilization-tube becomes more granular, stains more deeply, and acquires the character of a receptive spot. An elongated vacuole is often associated with the sperm-nucleus. In Fig. 25 this could be traced back into the fertilization-tube outside the oosphere. In Fig. 26, a case in which the fixing was not quite satisfactory, the vacuole-like structure occupies a tangential, not a radial, position. The limits of the cellulose cell-wall of the fertilization-tube at this time were not determinable with any degree of accuracy. The cellulose wall seems to stop short at the periphery of the egg, but the boundary between the ooplasm and the contents of the fertilization-tube is as represented in the figures, and gives the impression of a tube which penetrates some distance into the egg. Just at the time when the wall of the young oospore makes its first appearance, one frequently finds what appears as a prolongation of the fertilization-tube into the oospore in the form of a thimble-like vacuole (Fig. 28).

These facts, taken by themselves, might still fail to satisfy sceptics as to the existence of fertilization in the Saprolegnieae. Fortunately, the close study given to the sperm-nuclei resulted in a very unexpected and apparently, so far as plants are concerned, a unique discovery.

The centrosome and astrosphere of the male nucleus. The sperm-

nucleus soon after its entry into the oosphere, acquires a distinct centrosome and astrosphere. The centrosomes may have been present in the antheridia and fertilization-tubes in an unrecognizable condition, but the astrospheres are certainly new developments. The astral rays are always directed at first to the periphery of the oospore, as shown in Figs. 28 and 29, and they are associated with a mass of protoplasm having most of the characters of an ovocentrum. This mass of protoplasm might be regarded as homologous with the receptive spots found in the oogonia of the Peronosporae. Apart from the fact that it appears in *Achlya* at the periphery of the oospore and not of the oogonium, there is good reason for believing that its affinity is really with the ovocentrum. One might go so far as to call it a spermocentrum to emphasize this point, but for the reason that the structure in question is not situated at the centre of the sperm. Fig. 27 shows the male centrosome and its astrosphere, with and without the associated protoplasm—at *a* and *b*. The nucleus is indicated separately at *c* for the sake of clearness. The astrosphere of the male nucleus was seen by me first in relatively poor preparations, when it was so badly fixed and stained that it was impossible to convince Mr. Pole-Evans—a very keen-eyed observer—of its presence. The figures are drawn from preparations in which the astrospheres were very conspicuous. These male astrospheres, in good preparations, are very striking objects. Mr. Pole-Evans made a drawing of one so that I might be able to compare our observations. The drawings contained exactly the same number of main rays. An inexperienced observer was also asked to examine a section, and he noticed the rays of the astrosphere at once. I have not been able to discover any record of a similar observation of astrospheres in association with male nuclei in plants.

Typical centrosomes and astrospheres appear to be much more rarely developed in plants than in animals. In Zimmermann's ('96) *résumé*, if we exclude, as we must, those cases resting on the evidence of Guignard, a very small number of genera are recorded as possessing centrosomes. Centrosomes together with their astrospheres have, however, been demonstrated very satisfactorily in *Sphacelaria* by Strasburger and Swingle ('97), in *Fucus* by Strasburger ('97) and Farmer and Williams ('96, '98), in *Dictyota* by Mottier ('98 and '00) and Williams ('04), in *Corallina* by Davis ('98)—the centrosome being represented in this case by a somewhat indefinite centrosphere—in *Surirella* by Karsten ('00), in *Peziza*, *Ascobolus*, and *Erysiphe* by Harper ('95, '97), in *Agaricus* by Wager ('94), in *Pellia* by Farmer and Reeves ('94), and in *Fossombronina* by Farmer ('95). A number of genera might be added to these on the basis of older and, yet in many cases, apparently thoroughly trustworthy observations. The phenomenon appears to be commonest amongst the Thallophytes, especially among Fungi, but to be rare in vascular plants, possibly altogether absent.

Strasburger ('01) has expressed himself very strongly as to the improbability of their presence in the higher plants. It may be regarded as doubtful whether the blepharoplasts described by Belajeff ('98 and '99), Shaw ('98), Webber, Ikeno, and Hirase in various Vascular Cryptogams and Gymnosperms are the homologues of the centrosomes found in Thallophytes and liverworts. In any case a critical summary of the present state of our knowledge as to the occurrence and distribution of centrosomes in plants appears to be a desideratum. In the genera mentioned above the male gametes have been critically examined in *Fucus* alone, and fortunately by such expert cytologists as Strasburger, Farmer, and Williams. Strasburger, in particular, paid special attention to the possibility of the existence of a male centrosome and astrosphere, being fully alive to the significance of its presence or absence in view of the current theories of fertilization. The observations of Farmer and Williams ('96, '98) are of special interest. They state 'the passage [of the antherozoid] through the cytoplasm [of the oosphere] is extremely rapid, as is proved by the rarity of specimens which we have obtained which show the antherozoid between the egg periphery and the centrally placed nucleus. In its path through the cytoplasm it rather resembles a chromatophore, from which, however, its reaction to stain readily serve to distinguish it. It travels towards its destination *with its blunter end forward*, and no trace of cilia could be discerned when it was once inside the egg.' An examination of the excellent figures of these three observers leaves little doubt that the sperm-nucleus in *Fucus* is destitute of a centrosome and astrosphere.

Male centrosomes and astrospheres are well known in animal eggs, and are especially well developed apparently in Echinoderms. It is interesting to note that that rotation of the axis of the sperm-nucleus and consequent change in position of the astrosphere which has been so often described, and is very beautifully illustrated for *Toxopneustes* by Wilson ('97), does not take place in *Achlya*. During the progress of the sperm-nucleus to the middle of the oosphere it appears to keep its astral rays directed constantly towards the periphery. This behaviour corresponds to that described as occurring in *Fucus* by Farmer and Williams, making the necessary allowance for the absence of the astrosphere in the Alga. The male astrosphere certainly influences the female, for that appears to be repelled and to be driven thus from its central position in the oospore (Figs. 28 and 29). When the sperm-nucleus reaches the central region of the oospore, the dense protoplasm accompanying it goes to swell the ovocentrum, and this regains its original central position (Fig. 28). The ovocentrum now encloses two nuclei, two centrosomes, and two astrospheres.

Disappearance of the astrospheres and ovocentra and the fusion of the gameto-nuclei. The detection of the male astrospheres proved difficult.

The study of their fate has been practically a failure. They, in fact, soon disappear, and the mode in which this takes place has still to be determined. The ovocentra gradually disappear too, being however still clearly visible when all traces of the astrospheres have vanished (Fig. 30). The male and female nuclei increase in size and capacity for staining during this period, are easily demonstrable, and are always found in close proximity to each other near the centre of the oospore. When the ovocentra have finally disappeared, the gameto-nuclei may be found either close together or at a considerable distance apart, and frequently at some distance from the centre of the oospore (Fig. 31). In view of these phenomena it seems useless to discuss Boveri's theory of the function of the centrosome in fertilization. At any rate, it is only necessary to state that the fusion of the gameto-nuclei is delayed for at least twenty-four hours, and that the division of the zygote-nucleus may not take place for months.

It is, however, worthy of note that the nuclei at this stage are easily fixed and stained. Binucleate oospores representing this stage would be, in fact, the most obvious structures in moderately good preparations. This must be the stage which Humphrey and Hartog saw and figured as representing the last of the nuclear fusions assumed by them to take place in the oogonium and oospheres. I examined critically three oogonia containing respectively fourteen, thirteen, and fifteen oospores, and all were binucleate. Thousands of sections of oospores must have been casually observed in this condition whilst searching for other stages, and they were always binucleate or uninucleate. If uninucleate, one could confidently predict that an adjacent section would be likewise uninucleate. The binucleate character of isolated oospores has often been proved in this way. It is only after repeated experiences of this kind that one becomes confident that uninucleate sections always go in pairs.

This stage in development is, of course, preceded by one in which fertilization is going on, and if attention be directed specially to it, and the oogonia are carefully examined, we find that fertilized and unfertilized oospheres occur together in the same oogonium. We see, side by side, genuinely uninucleate and binucleate eggs, not simply uninucleate and binucleate sections. Such a condition of affairs depends upon the obvious fact that the fertilization of the numerous oospheres is not affected simultaneously. Three oogonia in this condition were examined critically. No. 1 possessed eight uninucleate oospheres and two binucleate oospores; No. 2 possessed one uninucleate oosphere and six binucleate oospores; No. 3 possessed six uninucleate oospheres and two binucleate oospores.

During the maturation of the oospores the oospore-wall thickens and granules of reserve material collect in the protoplasm. It is only when these processes are well under way that the fusion of the gameto-nuclei takes place. A remarkable feature of this stage is that the chromosomes

seem to be still recognizable, being grouped apparently in fours (Fig. 32). This reminds one of the statements of Berlese ('97) as to the number of chromosomes counted by him in the oospores of the Peronosporae under similar conditions—statements which certainly aroused considerable scepticism at the time they were published.

The further ripening of the oospores and their germination were not investigated in this species. The observations recorded suffice to prove once more the familiar succession in the number of the nuclei, the formula 1, 2, 1 being again demonstrated.

The number of chromosomes. As already indicated above, a study of the first mitosis in the oogonium led to the conclusion that the number of chromosomes in the nuclei was certainly more than four, and probably eight. The more difficult study of the second mitosis makes it pretty clear that the number of chromosomes is reduced therein to four. At metaphase in side views of the spindle we see clearly two or three chromosomes—numbers which would naturally occur if there were really four chromosomes in the nuclear-plate (Fig. 18 *k* and 20 *a*). It is not without significance, too, that four chromosomes can sometimes be seen in each of the conjugating gameto-nuclei (Fig. 32). Stress need not be laid on the actual numbers, as there are several sources of error in such determinations, but the reduction is definite enough. Such a division is to be regarded as a reducing division; a reduction in the number of chromosomes takes place in gameto-genesis in this plant as in most animals, and not in sporogenesis as in most plants. A detailed discussion of the physiological significance of such reductions may well be deferred until more information has been gathered as to their distribution amongst plants, and especially amongst the sexual and apogamous members of the *Saprolegniae*. The view expressed by me in '99 as to the significance of these divisions during gameto-genesis must, for the present, suffice.

Anomalies. In the discussion of cytological problems a consideration of typical forms is of much more importance than that of anomalous ones. The conclusions reached by the modern cytological methods become quite untrustworthy in the presence of many anomalies, for the simple reason that anomalous and typical forms get grouped together to form an unreal series—a sequence is evolved which is purely imaginary. One must constantly bear in mind that the series of stages in development described in such a paper as this are not consecutive stages in the development of some one individual organ, but rather stages arranged consecutively with more or less success, according to the calibre of the observer, representing a series of such organs. With this difficulty always in view, great care has been taken in the selection and cultivation of the material employed in my researches on the *Saprolegniae*. In certain preliminary studies, on material afterwards rejected, anomalies were only too abundant.

Two simple anomalies which came under observation in this species are, however, worthy of mention. In the case of one oospore—represented in Fig. 33—which contained a typical ovocentrum, an astrosphere and female nucleus, two sperm-nuclei had entered the oosphere through the fertilization-tube, and commenced to form astrospheres. The other oospores in the oogonium were normally fertilized. The fate of such sperm-nuclei is, of course, not ascertainable.

In one oogonium, containing about a dozen oospheres, three had the structure represented in Fig. 34. The impression given was that of two oospheres which had fused together, the line of fusion being indicated by the marginal indentations and the arrangement of the vacuoles in the plane connecting these. The nuclei, astrospheres, and ovocentra were well developed, and the mutual repulsion of these was very obvious. Such a case as this allows one to infer with some confidence that the size of the oosphere and the attracting (or repelling) forces associated with the nucleus and astrosphere stand in some definite quantitative relation to one another. The remaining oospheres in this oogonium were relatively small, but each had otherwise the normal structure. Although about thirty generations of cultures were examined in the fresh condition during the investigations made on this species, I never saw an oogonium with anomalous ripe oospores such as might be expected to result from the fertilization of such oospheres as these. It is upon such anomalous binucleate oospheres that Davis ('03) founds some of his most important conclusions. It would be useless to discuss these here. A theory founded upon exceptions and anomalies may be sound enough, but the exceptional facts recorded by Davis admit of a simple explanation, and certainly do not appear to have the phylogenetic significance which he attributes to them.

Summary of the evidence as to the occurrence of fertilization in the Saprolegnieae. In view of the facts recorded above, we may safely affirm that a normal fertilization takes place in *Achlya De Baryana*. For it has been shown that (1) the oosphere is uninucleate, the young oospore binucleate, and the old oospore uninucleate—the formula showing the number of nuclei in the reproductive organs is 1, 2, 1; (2) the protoplasm of the fertilization-tube becomes continuous with the ooplasm of the oosphere; (3) the entry into the oosphere of a sperm-nucleus can be traced; (4) the sperm-nucleus becomes associated with a special centrosome and astrosphere; and (5) the male and female nuclei fuse to form one zygote-nucleus. To *Achlya De Baryana* we may add, on the basis of my own earlier observations, fully concordant with the later ones so far as they go, four distinct species. The formulae as to the succession of the nuclei has been determined in these, and the cytological features are consistent throughout. The later results are, however, naturally more complete than

the earlier ones, as almost invariably occurs in all genuine investigations. The results in these four species are as follows:

1. *Achlya polyandra* has the formula 1, 2, 1, established in 1902.
2. *Achlya americana cambrica* „ 1, 2, 1, „ „ 1897.
3. *Saprolegnia dioica* „ 1, 2, 1? „ „ 1895.
4. *Saprolegnia mixta* „ 1, 1 or 2, 1 „ „ 1895.

Wager ('99) has confirmed the fundamental points of my earlier work. His formula would be 1, 2, x . He appears strongly inclined to the view that fertilization must take place, but expresses himself with commendable caution.

The negations of Hartog and Davis. Hartog apparently still adheres to his original view as to the wholesale fusion of nuclei in the oogonium, but since Davis has confirmed my results as to this point, and the above detailed observations show that no such fusions could possibly take place in these forms, there seems no adequate reason for subjecting his very pointed criticisms of my work to a detailed examination. The policy of appealing to fact, and of avoiding a war of words, finds ample justification.

Davis, however, is inclined to adopt Hartog's view—supported as it was a few years ago by the almost universal belief in the complete apogamy of the Saprolegnieae—that normal fertilization does not take place in this group. His contentions seem to me to be refuted by the facts described above, so that the necessity for a detailed examination of his criticisms likewise no longer exists. Moreover, the investigation of *Achlya De Baryana* having led to the discovery of the second mitosis, and of the centrosome and astrosphere associated with the sperm-nucleus in fertilization, it becomes necessary that Davis's species should be reinvestigated so as to test his view of the origin of the 'coenocentrum.' The question of the occurrence of a second mitosis in apandrous oogonia, and of the fate of the nuclei in these organs, forms a very difficult but interesting problem for future investigation—one, indeed, that should prove profitable in the highest degree.

CONCLUSION.

The problem of fertilization in the Saprolegnieae is, it seems to me, to a certain extent solved. Some species are typically sexual, others obviously apogamous. Between these extremes there are intermediate types. It is to be hoped that some of those able cytologists who have already done such good work on the Peronosporae will turn their attention to the Saprolegnieae. Those who take up the study of such forms as *Achlya De Baryana* and *Achlya polyandra* will soon find themselves in a position to

bear witness to the existence of fertilization in this most interesting group of Fungi. I venture to predict that the Saprolegnieae will soon furnish many more examples of typically sexual species.

SUMMARY OF THE CYTOLOGY IN *ACHLYA DE BARYANA*.

1. The nuclei in the oogonia and antheridia all undergo a first mitosis, the number of chromosomes being apparently eight.
2. Some of the daughter-nuclei in both oogonia and antheridia undergo a second mitosis in which the number of chromosomes is apparently reduced to four.
3. Centrosomes and astrospheres are developed in the second mitosis in the oogonia, but have not yet been observed in the antheridia.
4. The supernumerary nuclei in the oogonium undergo degeneration and absorption by the ooplasm. There are no nuclear fusions. The process of degeneration is virtually completed when 'balling' begins.
5. The supernumerary nuclei in the antheridia and fertilization-tubes degenerate much more slowly than those in the oogonia.
6. The 'origins' and oospheres are uninucleate. Each nucleus is associated with a well-developed centrosome and astrosphere, and an ovocentrum.
7. In fertilization, an open communication is set up between the fertilization-tube and the oosphere, and a single male nucleus accompanied with a gonoplasm passes out of the fertilization-tube into the oosphere.
8. The sperm-nucleus acquires, in the oospore, a centrosome and astrosphere, and as it moves towards the oosphere-nucleus, the centrosome and astrosphere are directed outwards. There is no rotation of the axis of the sperm-nucleus as in animal eggs.
9. The fusion of the gameto-nuclei does not take place until centrosomes, astrospheres, and ovocentra have disappeared. The male centrosome does not seem to have any special significance in the act of fusion or of the subsequent mitosis. Boveri's theory of fertilization does not seem applicable to plants.

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EXPLANATION OF FIGURES IN PLATES XXXIV-XXXVI.

Illustrating Dr. Trow's paper on Fertilization in the Saprolegnieae. Jm

All the figures were drawn with the help of the camera lucida. The finer details were of course filled in by freehand after the main outlines had been traced under the camera. All the nuclei present in a section were drawn, each generally in median optical section. The details of the cytoplasm are represented somewhat diagrammatically, as it is impossible to represent more than one optical section in the same drawing. All the sections figured were 5μ thick. Various stains were used. The drawings were all made under a magnification of about 1,250 diameters. Figures 1 to 13, which represent sections of *Achlya polyandra*, have been reduced during the process of reproduction to about 800 diameters. The remaining figures, which represent sections of *Achlya De Baryana*, have, with the exception of 14 and 15, not been reduced. Some of the drawings of the more typical nuclei, as well as other details in the various sections, have been enlarged and inserted separately in the plates in the neighbourhood of the sections to which they belong, so that the structure might be more certainly demonstrated.

Achlya polyandra.

Fig. 1. A somewhat tangential section of an oogonium with the nuclei in metaphase and anaphase. *a* and *b* are apparently undergoing a second mitosis.

Fig. 2. A nearly median section of an oogonium with the nuclei in mitosis. One nucleus showing the polar view of the nuclear-plate.

Fig. 3. A median section of an oogonium, and a section of an antheridium with the nuclei in mitosis. One nucleus has a typical spindle and nuclear-plate.

Fig. 4. One section (the fourth of nine) through an oogonium which contained four origins, each uninucleate. Three origins shown, each with a functional nucleus (*f.n.*) and degenerate nuclei (*d.n.*).

Fig. 5. Section of an oogonium (the first of eleven examined) which contained seven uninucleate oospheres. A doubtful centrosome is shown at *a*.

Fig. 6. The third section of the same oogonium (as in Fig. 5).

Fig. 7. The fifth section of a series of five sections through an oogonium which contained five binucleate oospores. *a* had two nuclei in the fourth section and *b* had one. Two other oogonia on the same slide had eight and six binucleate oospores in series of seven and eight sections respectively.

Fig. 8. A young oospore. The two nuclei in contact by their anterior pointed ends, where is to be seen a pair of deeply stained granules.

Fig. 9. One of four half-matured oospores present in a small oogonium, all of which were uninucleate.

Fig. 10. One of four sections of an oogonium which contained three uninucleate oospores. The oospore wall well developed and as thick as the wall of the oogonium.

Figs. 11 and 12. Sections (two of five) of an oogonium which contained two oospores only. The fertilization-tube in eleven is anomalous, the normal tube being shown in twelve. The swollen fertilization-tube is likewise, so far as my experience goes, a unique anomaly.

Fig. 13. Section of an oospore with fertilization-tube attached to it, the protoplasm in both being perfectly continuous.

Achlya De Baryana.

Fig. 14. Section of a young oogonium. Basal wall not yet formed.

Fig. 15. Median section of an oogonium and antheridium. All the nuclei in mitosis, excepting one or two which appear to be undergoing degeneration.

Fig. 16. Section of an oogonium with nuclei in mitosis. Nuclear figures somewhat irregular, suggesting a preparation for a second mitosis.

Fig. 17. Thin layer stage in development of the oogonium. The nuclei are either undergoing degeneration or are in the prophase of the second division. The two nuclei in the antheridium are in the metaphase stage of the first mitosis—one is seen in a profile, the other in a polar view. See text, pp. 553-4.

Fig. 18. 1+2+6+7+8+9+12+17 shows sections or portions of sections through one oogonium studied very thoroughly in a series of seventeen sections. The indices represent the numbers of the sections. Some of the nuclei are undergoing a second mitosis. The centrosomes and astrospheres make their first appearance at the poles of the spindles. Many nuclei are undergoing degeneration. See text, pp. 554-6.

Fig. 19. Section through an oogonium at the commencement of the formation of the 'origins.' Four origins are shown, each with a centrosome and astrosphere. A nucleus is present in each, too, but is only shown in *a*. There is a small supernumerary nucleus in *d*.

Fig. 20. Tangential section of an oogonium which contained four uninucleate 'origins.' At a lower focus the two origins of this section were continuous. The nuclei of the antheridium are in the metaphase of the second mitosis.

Fig. 21. Radial section of an oogonium with old origins. Mitoses completed in antheridia. Commencement of the formation of the ovocentrum. The nucleus has now no central chromatic mass. The centrosomes and astrospheres are very obvious structures.

Fig. 22. Small portion of an oogonium with the adjacent antheridium, drawn to show a typical oosphere. Seventeen similar ones were observed in a series of six sections, but fertilization-tubes were absent.

Fig. 23. Section of an oosphere at the moment of fertilization. A gonoplasm is present in the form of a thin strand which precedes the sperm-nucleus. The nucleus of the oosphere was in the adjacent section.

Fig. 24. Two serial sections of a small part of one oogonium showing the fertilization of one oosphere. The entry of the fertilization-tube and sperm-nucleus is shown in *a*. The median section of the oosphere is shown in *b*, the nucleus, however, not being inserted.

Fig. 25. Median section of a young oospore, showing female nucleus, centrosome, and astrosphere, the entry of the sperm-nucleus and the presence of a receptive spot.

Fig. 26. Portion of a section of an oogonium and antheridium showing the fertilization of two oospheres. In *a* the sperm-nucleus obscures the centrosome situated below or behind it. A portion of the ovocentrum is seen, but the female nucleus was in the adjacent section. In *b* the sperm-nucleus has apparently taken a tangential course through the oosphere.

Fig. 27. Tangential section of an oospore. At *a*, the male centrosome and astrosphere imbedded in the ooplasm; at *b*, the male centrosome and astrosphere without the ooplasm; at *c*, the associated male nucleus with the position of a portion of the astrosphere indicated.

Fig. 28. Fertilized oospheres, the gameto-nuclei being accompanied by centrosomes and astrospheres. Rays of male astrospheres directed outwards.

Fig. 29. Young oospores, in which the wall is clearly visible. The mode of attachment of the fertilization-tubes illustrated. The rays of the male astrospheres are directed outwards.

Fig. 30. Young oospore. The centrosomes and astrospheres have disappeared and the ovocentrum is relatively small, but still includes the two gameto-nuclei.

Fig. 31. Portion of a section of an oogonium with two binucleate oospores. The ovocentra have entirely disappeared, and the nuclei no longer occupy a central position. Deeply staining granules make their appearance in the protoplasm.

Fig. 32. Section through a portion of an oogonium showing three oospores with conjugating gameto-nuclei. The chromosomes of the nuclei appear to be still in evidence. The granules in the protoplasm are very conspicuous. In two of the oospores the nuclei are alone represented in full.

Fig. 33. Section through an oospore which contained two sperm-nuclei. The female nucleus was in the adjacent section.

Fig. 34. An anomalous binucleate oosphere, one of three discovered in one oogonium.

seem to be still recognizable, being grouped apparently in fours (Fig. 32). This reminds one of the statements of Berlese ('97) as to the number of chromosomes counted by him in the oospores of the Peronosporae under similar conditions—statements which certainly aroused considerable scepticism at the time they were published.

The further ripening of the oospores and their germination were not investigated in this species. The observations recorded suffice to prove once more the familiar succession in the number of the nuclei, the formula 1, 2, 1 being again demonstrated.

The number of chromosomes. As already indicated above, a study of the first mitosis in the oogonium led to the conclusion that the number of chromosomes in the nuclei was certainly more than four, and probably eight. The more difficult study of the second mitosis makes it pretty clear that the number of chromosomes is reduced therein to four. At metaphase in side views of the spindle we see clearly two or three chromosomes—numbers which would naturally occur if there were really four chromosomes in the nuclear-plate (Fig. 18 *k* and 20 *a*). It is not without significance, too, that four chromosomes can sometimes be seen in each of the conjugating gameto-nuclei (Fig. 32). Stress need not be laid on the actual numbers, as there are several sources of error in such determinations, but the reduction is definite enough. Such a division is to be regarded as a reducing division; a reduction in the number of chromosomes takes place in gameto-genesis in this plant as in most animals, and not in sporogenesis as in most plants. A detailed discussion of the physiological significance of such reductions may well be deferred until more information has been gathered as to their distribution amongst plants, and especially amongst the sexual and apogamous members of the *Saprolegniae*. The view expressed by me in '99 as to the significance of these divisions during gameto-genesis must, for the present, suffice.

Anomalies. In the discussion of cytological problems a consideration of typical forms is of much more importance than that of anomalous ones. The conclusions reached by the modern cytological methods become quite untrustworthy in the presence of many anomalies, for the simple reason that anomalous and typical forms get grouped together to form an unreal series—a sequence is evolved which is purely imaginary. One must constantly bear in mind that the series of stages in development described in such a paper as this are not consecutive stages in the development of some one individual organ, but rather stages arranged consecutively with more or less success, according to the calibre of the observer, representing a series of such organs. With this difficulty always in view, great care has been taken in the selection and cultivation of the material employed in my researches on the *Saprolegniae*. In certain preliminary studies, on material afterwards rejected, anomalies were only too abundant.

A.H. Trow del.

Huth, lith. et imp.

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A.H. Trow del.

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33.

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