The Karyology of Saprolegnia.

BY

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With Plates XXIV and XXV.
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I N the summer of 1893 I proceeded to Freiburg with a view to acquiring, among other things, a knowledge of certain of the newest methods in botanical technique. Prof. Oltmanns, who very kindly allowed me to work in his laboratory, and gave me, especially at the outset, much invaluable assistance, suggested that I should apply these methods to the detailed study of the cytology of the sexual organs of the Saprolegnieae. Humphrey's ('92) monograph on the group had just appeared, and it was evident that there were still considerable gaps in our knowledge, more particularly with respect to the behaviour of the nuclei in the gametangia. The work was subsequently carried on partly in England, partly in Germany, until in the summer of 1894 I obtained results which suggested further study, which has been prosecuted entirely in the Biological Laboratory of the University College, Cardiff.

The long series of researches by De Bary (commenced in '52 and terminated only by his death) and Pringsheim (com-

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menced in '51 and terminated in '83) not only made us well acquainted with the whole course of development in a considerable number of species of Saprolegnieae, but placed the taxonomy of the whole group for the first time on a satisfactory basis. The method adopted by De Bary of cultivating each species separately for many generations—that of so-called 'pure cultures'—has been of inestimable value.

It may be useful to briefly refer to those features in the structure and life-history of the Saprolegnieae which were placed beyond doubt mainly by the labours of these two great pioneers.

In the Saprolegnieae the mycelium is branched, unicellular and multinucleate,—or to use Vuillemin's term,—*apocytial*. Certain branches—the rhizoids—penetrate the nutritive substratum and serve to absorb nourishment from it; other branches, when fully developed, bear the reproductive organs. These are, with rare exceptions, of two kinds—asexual and sexual. The asexual organs are sporangia, and give rise to numerous biciliate zoospores. The sexual organs—oogonia and antheridia—are produced in considerable numbers. Oogonia have been found in nearly all the species, and it is by no means improbable that they occur even in those few species in which their presence has not, so far, been noticed. The antheridia may, according to the species, be always absent from the oogonia, always present on the oogonia, or present on some of the oogonia and absent from others.

The antheridia are generally borne on special antheridial branches. When these branches arise near the oogonium the species is said to be *androgynous*, and when at some distance from the oogonium, *diclinous*.

It is especially noteworthy that in two species only—*Achlya stellata* and *Saprolegnia monilifera*—have the oogonia been found to be invariably destitute of antheridia. The closely related species or races—*S. mixta*, *S. torulosa*, and *S. Thureti*—have a certain but variable number of oogonia with antheridia attached to them. In cultures of *S. mixta* 50% of the oogonia bear antheridia, in cultures of *S. torulosa* antheridia

are seldom to be found, while they have only been seen in *S. Thureti* on very rare occasions.

An oogonium produces from 1 to 40 or 50 oospheres. The antheridia give rise to small tubes—fertilization-tubes—which pierce the walls of the oogonium and apply themselves closely to the naked oospheres. The oosphere then becomes clothed with a wall of cellulose and is now known as an oospore. In the young oospore De Bary noticed a clearer central speck, which he looked upon, at least provisionally, as a nucleus. In the mature oospore an oleaginous mass, which generally occupies a central position, is universally present. The oospore which usually remains in the resting condition for some time, ultimately germinates, and produces in most cases a hypha, which either forms a sporangium, or at once develops into a new generation.

De Bary ('81) has given us an admirable account, with figures, of the stages in the development of the oogonium and oospheres in *S. Thureti*. The oogonium in the Saprolegnieae makes its appearance as a terminal swelling either on main or lateral branches, or occasionally is formed in an intercalary manner at some other point of a filament. Large quantities of protoplasm pass into the swelling, which is then delimited from the rest of the mycelium by one or two transverse walls. The mass of protoplasm generally has the form of a hollow sphere and surrounds a large central vacuole, which is continuous at first with the vacuole of the filament upon which the oogonium is borne. After a time, peculiar shifting vacuoles make their appearance in the protoplasm. These fuse together and finally unite with the large central vacuole. The layer of protoplasm thus becomes denser and thinner and the central vacuole larger. The protoplasm then moves gradually towards certain points so as to form hemispherical heaps projecting into the vacuole, and attached to one another ultimately by an extremely thin layer at their bases. These heaps may be called the oosphere-rudiments. Ultimately the thin connecting layer is ruptured and the oospheres lie free, in contact with each other, in the cavity of the oogonium. After a

certain amount of amoeboid movement, during which small portions of non-nucleated protoplasm are extruded from and again absorbed by them, the oospheres become perfectly spherical and are then furnished with a thin hyaline border.

In those species in which antheridia are produced, the antheridial branches attach themselves to the oogonium after it has attained its full size and before the formation of its basal wall. The antheridia are then separated off by transverse walls. The fertilization-tubes begin to grow out as soon as the oospheres have been formed.

It is obvious from the foregoing that it is very easy to identify, even in sections, a large number of successive stages in the development of the sexual organs—a matter of considerable importance in a study involving the accurate determination of the relative ages of structures which cannot be thoroughly examined in the living condition.

De Bary ('81, '83), influenced no doubt by the indubitable apogamy of the species destitute of antheridia, came to the conclusion that the Saprolegnieae as a group were distinctly apogamous: this conclusion was based on his inability to trace the passage of protoplasm from the fertilization-tube to the oosphere.

Pringsheim ('82, '83 *b*), as is well known, maintained that in certain species at least, fertilization took place.

I do not propose to enter into the merits of this controversy or to discuss the results obtained by other botanists [e.g. Zopf ('82, '83)]. I venture to believe that the methods at the disposal of these observers were not sufficiently accurate to insure trustworthy results. There seems also to be some doubt as to the identity of the appearances described by Pringsheim and Zopf. In this paper, however, I hope to prove conclusively that the genus *Saprolegnia* contains at least two species (*S. dioica* and *S. mixta*) in which fertilization either invariably or frequently takes place, and have little doubt that two species of *Achlya* may be added to the list. It is well to lay stress upon this at the outset, since under the influence of the great name of De Bary, the

apogamy of the Saprolegnieae has been generally accepted by botanists.

It is only in recent years that the karyology of the group has received much attention. In addition to the researches of Schmitz ('79) and Strasburger ('80), special studies of the structure, behaviour, and fate of the nuclei in these plants have been made by Hartog ('89, '91), Dangeard ('90 *a*, '90 *b*, and '91) and Humphrey ('92).

Hartog ('91) states that the 'nuclei are vesicles with a central chromatin-mass supported by a network of "linin" or nucleo-hyaloplasma.' The nucleus of the zoospore is spherical and undergoes divisions by a process which is virtually direct, but nevertheless exhibits some features of karyokinesis. He ('89) also states that the division of the nucleus is preceded by great changes in the central mass of chromatin. This becomes very irregular and ends by dividing into two halves having the form of blunt crescents, placed back to back, and in structure distinctly 'fibrillaire.' These crescents move away from each other (*s'écartent*) and become spherical, and finally the nuclear wall is inflected so as to separate them, and thus the two daughter-nuclei are constituted. The nuclear divisions go on only in the mycelium and antheridia. No divisions consequently take place in the sporangia and oogonia. The number of nuclei which passes into a sporangium is just sufficient to provide one for each zoospore. A large number of nuclei pass into the oogonia—many more than is sufficient to provide one for each oosphere. The vacuoles which form in the young oogonia bring these nuclei nearer to each other, and they fuse in pairs until, in the genus *Saprolegnia*, the number is reduced at the time of the formation of the oospheres to one for each oosphere. Some of the details of the fusion are particularly interesting. 'In fusion of the nuclei the chromatin-masses long remain distinct, but are *smaller and take up stain less readily* (the italics are mine), and the nuclear wall at this stage ceases to stain, so that the fusion-nuclei have the look of vacuoles in the cytoplasm.' In his last paper Hartog carefully distinguishes between these

vacuole-like fusion-nuclei and the true vacuoles. He comes to the conclusion that the male gametangia are functionally impotent, and has developed a theory to explain his observations which would be all the more interesting and valuable if these were entirely beyond question. 'We have,' he says, 'a case of multiple endogamous union of potential gametes.'

Hartog further points out that the young oospore is uninucleate in *Saprolegnia*, but frequently binucleate in *Achlya*. He has apparently made no observations on the germinating oospores, and, so far, has unfortunately given no figures.

Dangeard's ('90 *a*) results are in accordance with those of Hartog up to a certain stage. The differences on the really critical points, however, are very great. This author, putting aside the question of vacuoles, finds no evidence of fusion-nuclei. He says, that from the vacuolated stage onwards to the formation of the young oospore, one sees only fine granules of chromatin dispersed in the protoplasm,—'la substance des noyaux s'être éparpillée'; and also asserts that the ripe oospore is multinucleate, but as to the origin of these nuclei he can only offer conjectures.

Dangeard suggests, in addition, that the 'Kern-fleck' of De Bary seen in the young oospore is the commencement of the fatty mass and not a nucleus at all, and that certain small deeply staining bodies which make their appearance in the young oospore are globules of glycogen. It is well to pay some attention to these curious and bewildering observations,—on which I hope to be able to throw some light—as they so plainly indicate the special difficulties of the investigation. Dangeard leaves the question of fertilization undecided.

Humphrey's ('92) conclusions do not differ to any great extent from those of Hartog. In particular, both of these botanists are agreed as to the reduction of the nuclei in the oogonium by a process of fusion, the uninucleate character of the ripe oospores, and the impotency of the antheridia. Humphrey, however, finds no evidence of division of the nucleus taking place in the antheridia. His figures, of which comparatively few deal with the histology, are of very con-

siderable interest, but furnish no real evidence of the existence of fusion-nuclei. The figure of a binucleate oospore of *Achlya americana*, together with Hartog's statements concerning another species of *Achlya*, are the grounds for my belief that fertilization takes place in *Achlya* as well as in *Saprolegnia*.

My own studies have led me to conclusions which differ fundamentally from those advanced by these three observers. The results brought forward in the present paper will, I trust, help us to form definite conclusions respecting these divergent and strikingly contradictory views.

In his investigations, Humphrey adopted the method of imbedding in paraffin and cutting serial sections with the microtome. His material was stained *en masse* by haematoxylin and then imbedded and sectioned. I have tried this method, but have not been able to obtain good results with it. Hartog and Dangeard have apparently trusted to preparations involving fixing, staining, clearing and mounting only. Such methods may suffice to elucidate the structure of the zoospores and mycelium together with their nuclei, but it appears to me to be totally inadequate to solve the difficult problems presented by a study of the sexual organs. Hartog's observations, nevertheless, so far as they go, appear to be most accurate and reliable, and it is only in the interpretation of them that I differ widely from him.

The question of the formation and liberation of the zoospores, which since 1880 has attracted the attention of a very large number of botanists, cannot be entered into here. The figures of sections of sporangia here given are intended only to illustrate the behaviour of the nuclei.

METHODS.

As the genus *Saprolegnia* is admirably adapted for teaching purposes and has been strangely neglected by the writers of text-books, it may serve more than one useful purpose if a somewhat extended account be given of the methods adopted in this research.

Cultures. It is obviously advisable to secure material which consists of a single species only. To obtain such, following in the main the method of De Bary ('88), samples of various aquatic plants, e.g. Algae, Mosses, Phanerogams, &c., are first collected from any convenient piece of fresh water. These are placed in small glass jars—marmalade pots answer well—and dead house-flies, previously wetted with alcohol and washed with water, are thrown on to the surface. Almost invariably some member of the Saprolegnieae is found growing on the flies in the course of two or three days. The fungus produces a kind of halo of hyphae extending all round the submerged parts of the fly's body. The sporangia, which are large enough to be detected by the practised unaided eye, rapidly make their appearance; and later on, generally in about a week, the sexual organs are formed.

When the sexual organs have produced ripe oospores, the purity of the culture may be roughly estimated. As a rule more than one species is present. If a suitable species has been found, it may be separated from those associated with it in several ways. The most effective method is to cut off, on a cover-glass, a small branch bearing oogonia; wash away all spores and extraneous matter with water sterilized by boiling; add the leg of a fly and a drop of water, and invert over a perforated piece of cardboard, which is made to rest on a glass slide and is kept thoroughly moist. The culture started thus, in a very simple and effective moist chamber, is kept under microscopic control for a day or two. The separated branch is not seriously injured by these manipulations, and generally puts out small branches which develop rhizoids and attack the leg of the fly. When it has been ascertained that the severed branch alone has infected the leg, cultures may be proceeded with on a large scale, as follows:

Glass jars are sterilized by a dilute solution of mercuric chloride and thoroughly rinsed with water carefully sterilized by boiling. Sterilized water must naturally be used for the culture-fluid. If small glass squares are used to cover these jars, a large number may be prepared at one time and kept

ready for use. Flies killed by chloroform to prevent injury to their chitinous coats, moistened with alcohol and washed with sterilized water, are next thrown into the jars. The infected leg of the fly is then taken and placed in contact with one of the floating flies. In a week the flies are well covered with so-called pure cultures of the species selected. The cultures are not, however, pure, as they contain bacteria, monads, infusoria, &c., and in specially hot weather these unwelcome organisms affect more or less unfavourably the healthy development of the cultures.

Successive generations may readily be produced by infecting a second jar with water from the first, a third with water from the second, and so on. To attain certainty as to the purity of a culture, it is well to keep it under microscopic control for several generations, and to adopt the usual precautions with respect to the sterilization of the various instruments used in the different operations. De Bary was able to maintain the purity of a culture of *S. Thureti* for a period of eleven years. I have obtained a pure culture by placing with the leg of a fly, as described, a single sporangium in which the spores were already outlined. The spores germinated *in situ*, infected the leg, and produced the required cultures. Cuttings of the hyphae behave in the same way.

On one occasion I succeeded in making a pure culture of *Aphanomyces laevis* from a single zoospore. The method adopted in this case may be of use later in determining the real nature of the diclinous species: it is certainly possible that these are dioecious. White of egg was beaten up and a small drop spread thinly on a cover-glass, which was then immersed in boiling water in order that coagulation might take place. The thin layer of albuminous matter is sufficiently transparent for observations to be made through it, and it provides suitable and abundant nourishment for the developing plant. These covers are then floated on the surface of the water in the jars containing a mixture of species of Saprolegniae. Of ten covers floated on the surface of a jar, nine were found after a day or two to be unaffected, the tenth had

a single spore upon it which had already commenced to germinate. The culture was continued in a moist chamber and observed day by day until the sporangia characteristic of *Aphanomyces* and the sexual organs characteristic of *A. laevis* were produced in succession.

It is worth mentioning that I have found hard-boiled white of egg, cut up into minute oblong pieces and floated on the surface of the water in the jars containing the cultures, to be a very good substitute for flies. At the commencement of the investigation, most of the cultures were carried on on meal-worms; later on white of egg was tried and used, but finally house-flies were found to be, upon the whole, the most satisfactory: if kept dry, as Humphrey ('92) has pointed out, they retain their nutritive properties for at least six months.

The vitality of these cultures is great: they may be neglected for six months, and still on adding fresh food-material, new growths at once appear. In the winter of 1894-1895 some of my jars containing cultures of *S. dioica*, which stood near an exposed window, had the water which they contained converted into solid masses of ice, but nevertheless fresh cultures were obtained from them when the ice melted.

The species I have principally examined in pure cultures and in the fresh condition have been *S. dioica*, De Bary, *S. Thureti*, De Bary, and *Achlya prolifera* (Nees, 1823), De Bary. I have carried the study no further than to satisfy myself of the correctness of De Bary's descriptions and to learn to discriminate the various stages in the development of the sexual organs for use in the detailed study of serial sections: such studies of fresh material are easily carried out in moist chambers by means of small cultures on the legs of flies. I have found that by changing the water the whole course of development (excepting naturally the germination of the oospores) may in this way be followed on a single culture.

The four species mentioned do not, however, include all those which I have been able to identify. *Apodya lactea*

(Agardh, 1824), Cornu, was found very unexpectedly, apparently associated with no other species, in a test experiment as to the purity of the Cardiff Water-Works water. I have seen most of the forms (or species) of the *Ferax*-group of De Bary, but have not been able to study them critically.

Fixing. Most of the better known methods of fixing were tried, viz. solutions of osmic acid, picric acid, and chromic acid, absolute alcohol, as well as a hot saturated solution of mercuric chloride—the last being recommended by Hartog and found most satisfactory by Humphrey. I have found Hartog's method to be the best, and have used absolute alcohol side by side with it, as a means of avoiding any error due to the effect of the fixing liquid.

Imbedding and Sectioning. The fixed material was generally washed for twenty-four hours in running water, and in the earlier experiments was passed into absolute alcohol by means of Schultze's dehydrating apparatus, but later on this was dispensed with. Graded alcohols give results which are sufficient for all practical purposes. The dehydrated material was passed into paraffin, chiefly through the medium of xylol, but sometimes of chloroform. The position of the fly and fungus is well seen in the paraffin. The fly was generally bisected longitudinally, certain useless portions—determined by preliminary experiment—cut away, and the remainder sectioned by means of a Jung microtome. Some of the sections were of 7.5μ thickness, a few of from 2 to 3μ , but most of them of 5μ . For the more difficult work, the last-mentioned thickness is very convenient: one gets by this means about four sections through the young oospore. I may add that the chitinous coat of the fly is easily sectioned, but it is better to cut it away so as to save the edge of the knife and avoid the sectioning of useless material. Moreover, the smaller the surface of section, the longer becomes the series mounted on the same slide—an advantage of some importance.

Staining. In the earlier experiments an attempt was made to stain the material *en masse* with haematoxylin. The

results, so far as concerns the nuclei of the zoospores and mycelium, were satisfactory, but the study of the sexual organs could not be carried out properly by this method. The staining was effected on the slide in all later experiments, and the advantages of this method compensate for its laboriousness: the stain comes into direct contact with the protoplasm and does not have to pass through more or less impermeable membranes.

The sections were fixed to the slide with a mixture of white of egg and glycerine, freed from paraffin and stained with Schneider's acetic carmine, gentian-violet and eosin, or Kleinenberg's haematoxylin, and all three methods gave excellent results. I have used the two former very extensively; the latter has furnished me occasionally with beautiful preparations. The best haematoxylin-stained preparations were obtained by using a very dilute solution, allowing it to act for twenty-four to forty-eight hours, and then partly decolorizing with acid alcohol, alum solution, or best of all by a saturated solution of picric acid. [See Zimmermann ('92).] Schneider's acetic carmine was diluted so that the solution contained from two per cent. to five per cent. of acetic acid, and the objects remained in the stain for from twelve to twenty-four hours.

For a long time I failed entirely to apply Gramm's method of double staining with gentian-violet and eosin, although I had been using it successfully for twelve months in the laboratory for a great variety of purposes. In the case of material fixed by absolute alcohol, I believe it is impossible to secure good results, as the gentian-violet taken up by the nucleus is almost instantaneously taken out again by the absolute alcohol used in dehydration. With material fixed by mercuric chloride, the same difficulty presents itself, but not to such an extent as to make it impossible to surmount. When successful, the preparations are so excellent that for most purposes they are far superior to those produced by any other method. The difficulty of carrying out this method satisfactorily is great, and at first only a small percentage of

the slides were satisfactorily stained : towards the close of the work, however, I succeeded in obtaining uniform results.

Simultaneous fixing and staining by picro-nigrosin can readily be carried out, and may be recommended for demonstration purposes ; this method does not appear, however, to be suitable for detailed critical study.

KARYOLOGY.

At the commencement of my investigation of the karyology of the genus *Saprolegnia*—the main object of the research—I devoted myself to the examination of pure cultures of *S. dioica*, De Bary—a diclinous species whose histology had till then, apparently, never been studied. In the summer of 1894 I was able to prove to my own satisfaction, and to that of Prof. Oltmanns, that fertilization took place regularly and normally in this species. In view of the controversies which have so long raged over this point in the Saprolegnieae, we both thought it advisable to make a special study of a species destitute of antheridia. I soon obtained pure cultures of *S. Thureti*, De Bary, which is, for all practical purposes, destitute of antheridia. After maintaining the purity of this culture under microscopic control for about six generations, large quantities were grown, fixed, and preserved in the usual way. These were not previously examined microscopically. I depended entirely upon macroscopic examinations with respect to the determination of the condition of the cultures, and that of course with a view to avoiding the handling of the specimens as much as possible, and thus securing better results. The first sections examined revealed the presence of oogonia with and without antheridia. Suspecting that in some inexplicable way the cultures had become impure, I re-examined them in the fresh condition, and found that the cultures now appeared to be a mixture of *S. Thureti*, De Bary, and *S. mixta*, De Bary. These forms are so closely allied, that but for the fact that De Bary found the first to maintain its characters for eleven years, and the second for five

years, I should have at once concluded that both species are simply forms of one produced by somewhat different external conditions of life. Under the circumstances, however, I concluded, at least provisionally, that the cultures were impure, and that the oogonia with antheridia might reasonably be referred to *S. mixta*, and the oogonia without antheridia to either *S. mixta* or *S. Thureti*. These are, of course, respectively *androgynous* and *completely apogamous* forms.

It would have been obviously entirely outside the province of this research to attempt the decision of the specific identity of *S. Thureti* and *S. mixta*; the work would probably have required many years to carry out, and the results would not have had, so far as I can see, the slightest effect in the solution of the problems investigated in this paper. As a matter of fact, it may be regarded, apart from the difficulties of description, as a fortunate circumstance that the material contained the antheridium-bearing form.

In the following descriptions I shall refer to the antheridium-bearing oogonia as those of *S. mixta*, and to the others as those of apogamous oogonia of *S. Thureti* and *S. mixta*.

The most beautiful results were obtained during the investigation of *S. mixta*, but simply because by this time the method of staining adopted had reached its highest degree of efficiency. So far as I am able to judge at present, the karyology in the three forms, with one important exception, is identical. Most of my figures, however, refer to *S. mixta*, for the reason already stated.

The zoospore. Structure of the nucleus. Zoospores which have come to rest and clothed themselves with a cell-wall are generally abundant in the preparations. They fix well and stain easily, and I have consequently come to regard them as a kind of index to the value of the preparations. Each zoospore contains a quantity of granular protoplasm which is of a more or less spongy texture. The granules or microsomes are frequently of considerable size, and stain readily, although not with so deep a colouration as the nucleus. The nucleus is spherical and is bounded by a distinct nuclear wall.

In the centre of the nucleus is a spherical mass, which in deeply stained preparations appears homogeneous, and has, I believe, always been figured as such. This may be regarded as a chromosome. Dangeard looks upon it as a nucleolus. In good preparations its substance is seen to be distinctly spongy; median optical sections, in particular, have the appearance of a network. The cavities of the meshes appear to be filled with a substance which has not the same affinity for nuclear stains as the substance which forms the boundaries of the meshes. Although it is a difficult matter to decide, I think that the strands of this meshwork never become resolved into threads or rods of any kind: it cannot consequently correspond to the nucleus of higher plants. The chromosome is apparently suspended in the middle of a nucleo-hyaloplasm—which occupies the space between it and the nuclear wall—by a number of threads, which are only distinguishable from the nucleo-hyaloplasm which they traverse, and of which they probably form a constituent part, by their reaction towards stains. It is frequently difficult to detect these threads, even in preparations which are otherwise good, and a detailed study of them has not been possible with the lenses at my disposal: they appear most clearly and constantly in the acetic carmine preparations. The zoospore generally contains a somewhat large vacuole.

In the young zoospores, before germination begins, one finds in the best preparations a number of granules close underneath the external surface. Two of these are frequently elongated and arranged as if constituting a pair, and I have consequently been inclined to regard them as having some connexion with the cilia. They stain deeply with nuclear stains and are represented in Fig. 1 *a*, between the nucleus and the vacuole.

Division of the Nucleus. Germination. Development and Structure of the Mycelium. Germinating zoospores frequently appear in the preparations, and it is by no means uncommon to find them conveniently sectioned in the plane of the germ-tube. The nucleus first undergoes division, and the division,

so far as I have been able to observe, *always* follows the direct type: my observations on this point, however, might well have been more extensive. The chromosome loses its spherical character, becomes oblong, and soon after appears to have a slight median constriction. The two halves thus outlined gradually move apart until they are separated from each other by a distance equal to about the diameter of the originally spherical chromosome. The nucleus as a whole increases in size and alters in shape to accommodate itself to these internal changes. The nuclear wall, however, remains intact. Finally, a new nuclear wall appears in the plane of the equator of the nucleus, as is shown in Fig. 1 *b*, and the division is nearly complete. This wall later appears to split into two lamellae, for a constriction of the nucleus takes place in its plane and thus brings about a complete separation of the two daughter-nuclei. Further divisions follow, and while this is going on the germ-tube is developed. The apex of the germ-tube appears at all times to be destitute of a nucleus (Fig. 1 *c* and *d*).

If we turn now to the study of the mature hyphae, sections of which abound everywhere in the preparations, we find that the nucleus is generally more or less elongated, and the chromosome alters its shape to keep pace with the change in the nucleus. The protoplasm forms a lining to the cell-wall, and encloses a large central vacuole, across which thin bridges or connecting strands of protoplasm sometimes stretch. The nuclei are fairly evenly distributed, but are sometimes to be found in groups in accumulations of the protoplasm. Divisions appear to take place regularly (except perhaps in those hyphae poor in protoplasm), and sometimes one sees very elongated nuclei like those in Fig. 2 *b*, as if a simultaneous division into more than two daughter-nuclei were to follow.

Sporangia. Formation of Zoospores. The phenomena connected with the development of the sporangium and spores appear to be mainly of physiological interest. A hypha is arrested in its growth, and considerable quantities of protoplasm pass into its apical portion. This apical portion filled

with dense protoplasm is the young sporangium. It becomes delimited from the mycelium by a cell-wall—the basal wall—and there is not the slightest doubt that the protoplasm at this stage contains the number of nuclei necessary to furnish each zoospore with a single nucleus. Fig. 3 *a* illustrates a transverse section of a fairly old sporangium with the zoospores nearly mature, and Fig. 3 *b* and *c* illustrate radial and tangential sections respectively of the lower portion of a younger sporangium. These figures will suffice to indicate how little need there is to dwell on the morphological characters. The nucleus, it will be noted, has again become spherical. In Fig. 3 *a* the zoospore-rudiments are almost separate; they are connected together merely by very thin strips of protoplasm which ultimately get ruptured and so give rise to the spores. In Fig. 3 *b* and *c* the protoplasmic bridges, connecting the young spores, which have been so frequently described, are well seen. It may be worth while to note that the sections appear to prove conclusively, if this were any longer necessary, that there is here no possibility of the formation of a nuclear or cell-plate or special expulsive substance. I have examined with some care sections of younger and older sporangia, the latter with the zoospores fully formed; but these furnish no additional features of interest.

Development of the Oogonium up to the complete formation of the Oospheres. The development of the oogonium has been most successfully followed in *S. mixta*, but there is no reason to believe that the other forms do not agree with this one in every respect. When an oogonium is formed at the end of a hypha, the latter ceases to increase in length, but grows in breadth, so as to give rise to a more or less spherical head. Large quantities of protoplasm and numerous nuclei pass into it. Fig. 4 illustrates a median section of a young oogonium which at this stage contained seventy-two nuclei: it is to be noted that the vacuole of the hypha extends into the oogonium. A careful examination of the nuclei shows that their structure alters coincidentally with their passage into the oogonium. In the hypha below the swelling the nuclei have the normal

vegetative structure. In the swollen portion the chromosome has become irregular in shape, and the nucleo-hyaloplasm takes up stains readily. The chromosome, with the double stain used, becomes violet, and the nucleo-hyaloplasm a dull red. The nuclear wall is extremely well defined; indeed the nuclei at this stage are very easy to demonstrate.

When the oogonium has attained its full size, the antheridia come into contact with it, and the basal wall is then formed. Fig. 5 represents this stage, and shows clearly that the nuclei are still in the same condition as that shown in Fig. 4. The number of nuclei in this particular oogonium was very small, and they were also of unusually large size. At this stage, the oogonium may, as figured, have no central vacuole: in by far the largest number of cases, however, a central vacuole appears to be present; and at all later stages up to the formation of the oospheres this is particularly evident.

Soon after the basal wall is formed, the nuclei of the oogonia undergo great changes which are extremely difficult to follow except in excellent preparations. As these changes have been either overlooked or partially overlooked, and entirely misinterpreted by all previous observers, I must here devote special attention to them. Careful and detailed examination of the nuclei shows that the microsomata in the protoplasm bordering on the nuclear wall become more deeply stained and conspicuous. The chromosome-mass undergoes division into two generally more or less unequal parts, and this process appears to take place, as represented in Fig. 6 *a*, by the appearance of a large cavity in the spongy mass immediately under the surface, near to which, therefore, it is thus bordered by a thin membrane only. The thin part appears to be ruptured, and the splitting commenced in this way is continued until the chromosome has been divided into two rod-like halves. Fig. 6 *b*, *c*, *d*, and *e* is very instructive. In *d* the rods produced by the division are shown in plan, in *e* in profile. Fig. 7 represents one of the sections of the oogonium from which these figures were drawn.

Somewhat older oogonia show that the rods escape laterally

from the stained microsomata and nucleo-hyaloplasm which surrounds them, and thus come to lie free in the protoplasm. At this stage no nuclear wall can be detected, although there is a clear space around the pair of rods: in the figures the boundary of this space is represented by a line, which must not be regarded as implying that the nuclear wall persists, though no longer evident. The evidence rather points to the conclusion that the nuclear wall is ruptured, and that the stainable nucleo-hyaloplasm, together with the deeply-coloured microsomata which surround the nucleus, are dispersed in the protoplasm, retaining however, for a time, their capacity for absorbing nuclear stains. These points will be appreciated so much the better if Fig. 8 *a, b, c*, and *d*, and Fig. 9 be consulted, the latter of which represents one section of the oogonium and antheridia in which these phenomena were specially studied. In Fig. 8 *a* the stained mass (nucleo-hyaloplasm, &c.) still lies in contact with the rods; in *c* it has entirely disappeared; and in *d* it seems further as if one of the rods were undergoing degeneration, as it does not stain deeply like its fellow. It will be noted, too, that the rods ultimately assume a spherical form.

Fig. 10 *a* and *b* deserves special study, as that of a stage immediately following upon the one represented in Fig. 9. Four of the pairs of now spherical rods are still to be seen, and are stained deeply; three of the pairs have undergone complete division so as to give rise to six new small daughter-nuclei, which are still, however, arranged in groups of two; while in addition there are six small solitary nuclei. One frequently notices in preparations of this stage that one or both of the paired rods stain badly; and occasionally, as at *deg. n.* in Fig. 10 *a*, we can just discern pairs in which the half-chromosomes have become so excessively small as to be scarcely recognizable. The stained nucleo-hyaloplasm and microsomata are still to be recognized, associated with those nuclei that were later than the others in entering upon division.

It would be natural to anticipate that, in consequence of

this division, the number of nuclei in the older oogonia would be doubled, since every nucleus in the oogonium undoubtedly passes through all the stages of division up to the halving of the chromosomes and the passage of the halves in pairs into the surrounding protoplasm.

A study of a somewhat later stage—the typical vacuolated stage—shows us clearly that the conditions are not so simple. Fig. 11 *a* and *b* represent two sections of an oogonium, one tangential and the other median, the greatest diameter of which was $50\ \mu$. The nuclei in this oogonium, which were all small, were counted carefully, and numbered forty-five. The oogonium probably contained at the outset, when the basal wall was formed, considerably over 100 nuclei: it should be remembered that seventy-two were counted in an oogonium with a diameter of $29\ \mu$. In consequence of the division the number would be doubled, but the number actually determined was forty-five. This, in itself, would be sufficient, provided the case were a normal one, to prove that a larger number of the small nuclei must undergo degeneration. Repeated examinations and measurements have convinced me that the case is a normal one. We have, however, ocular demonstration of the degeneration in the sections themselves; for the small and badly stained chromosomes which make their appearance while the division is still incomplete, must be looked upon as more or less degenerate. There is not the slightest evidence for the view that the reduction is brought about by nuclear fusions.

The significance of this division must, I think, be apparent to every one. It is exactly of the nature of a reducing division. The whole chromosomes are converted into half-chromosomes, which do not, as in the case of the direct divisions, become whole chromosomes by a process of growth.

These processes correspond very closely to the karyokinetic phenomena described by Hartog as occurring in these plants. Apart from referring to the fact that the evidence for the occurrence of indirect division of the nucleus has been made more complete by my observations, I need only state that

the points of difference between us are trifling, and concern the behaviour of the nuclear wall, which Hartog considers to remain intact, and the position of the half-rods back to back, and not face to face, as I have found them. As he expressly states that divisions do not occur in the oogonia, I conclude that he observed these phenomena in the antheridia, where they certainly occur regularly.

It is clear then that by the time typical vacuolization has begun, a reducing division of the nuclei, and the degeneration of the large majority of the daughter-nuclei thus formed, have taken place in the oogonium. The fact that it is the smaller half-chromosome that generally undergoes degeneration, and that the nuclei are slightly larger on the average at a later stage, leads me to believe that it is the smaller nuclei that go to the wall in this struggle for the survival of the fittest.

But an oogonium of 50μ diameter does not produce forty-five oospheres, and as the oospheres are uninucleate, it is clear that we have to explain how the number of nuclei is still further reduced. The vacuolated stage of the oogonium passes away and is replaced by one in which we have a thin layer of protoplasm lining the wall and surrounding the vacuole. Fig. 12 *a* and *b* represent two out of six sections, 7.5μ thick, of an oogonium whose greatest diameter measured about 45μ . The nuclei which could clearly be distinguished by any definite recognition marks numbered forty-one. Of these seven were noted as typical and thirty-four as degenerate. Three typical ones are shown in Fig. 12 *b* and twelve degenerate ones in Fig. 12 *a*: an oogonium of this size would produce about seven oospheres. The typical nuclei have the structure already described. The chromosome is small and its spongy character cannot be determined, simply because in order to see the nucleus at all it is necessary to stain as deeply as possible. The degenerate nuclei present various appearances: some of them stain so badly that they are not readily visible, and can be identified only by careful comparison with more deeply stained ones. The deeply stained nuclei have the appearance

of being made up of a number of much smaller ones, or sometimes appear distinctly spongy, and one can therefore readily understand how an observer might be tempted to conclude that they are fusion-nuclei. I believe that the granulated and spongy appearances may be traced to the same cause—the expansion and breaking up of the spongy network of which the chromosome-mass consists. The appearances represented in Fig. 12 *a* are then to be regarded as due to nuclear degeneration and not to nuclear fusions.

The protoplasm of the thin layer soon begins to be heaped up at certain points in order to constitute the oosphere-rudiments: Fig. 13 *a*, *b*, and *c* show an early stage in the development of these. In the three successive sections figured, three distinct rudiments will be noted. In each rudiment *there is one typical nucleus and one only*, having the structure we have observed in previous stages. Associated with it are degenerate nuclei, and some of these *do really appear to unite in pairs*: five distinct pairs may be noticed in the three sections. Not only do the forms assumed by these nuclei suggest fusion, but the increase in size and decrease in number is very marked. I believe that much of the confusion which has crept into the study of apocytial plants can be traced to the fusion of these degenerate nuclei, together with the study of badly stained preparations. These abnormal nuclei ultimately undergo complete degeneration, becoming more or less deformed, and finally losing all capacity for taking up nuclear stains. I have never been able to discover them in fully formed oospheres, but they are frequently still noticeable as irregular bodies near the surface of the older oosphere-rudiments.

Fig. 14 shows one of four sections through a small oogonium 28μ in diameter, which contained four oosphere-rudiments, each uninucleate like that figured. The perfect agreement in structure of this nucleus with the typical ones seen in Fig. 13, and the complete dissimilarity from the degenerate nuclei, leave no room for doubt as to which are the functional nuclei in the younger oogonia.

When the oospheres are fully formed, we find, as represented in Fig. 15, that the nucleus still persists, but has undergone slight modification—the chromosome-mass has become more or less irregular. This irregularity of the chromosome left me for a long time in doubt as to whether fusions might not actually take place in the case of the functional nuclei at a very late stage, and I was especially anxious to determine this point in the apogamous forms. I have at last convinced myself that from the inception of the oogonium to the complete development of the oospheres, *no fusion of functional nuclei takes place.*

Figs. 16 and 17 show clearly that both young and mature oospheres of *S. dioica* are uninucleate, and the small size of the nuclei and general behaviour leave no doubt in my mind of the occurrence of reducing divisions in this species also. Similar evidence may be brought forward with respect to the apogamous oogonia of *S. Thureti* and *S. mixta*.

I cannot but conclude, however, that these observations will meet with considerable adverse comment, and it will be as well to bring forward at this stage additional independent evidence in support of them.

Hartog, who admits that divisions take place in the antheridia, denies that they take place in the oogonia. All observers are agreed that the oogonia and antheridia are homologous organs. They represent the culminating point of a development, which, starting with typical isogamy, terminates in complete heterogamy. Such developments are obviously of secondary importance, but reducing divisions must be of primary value, as they of necessity date back to the evolution of the sexual condition itself. If the nuclei of the antheridia undergo division, it is extremely improbable *a priori* that the nuclei of the oogonia should remain undivided.

Wager ('89) has carefully investigated the karyology of *Peronospora parasitica*, and has described and illustrated with numerous figures a process of karyokinesis which, while taking place in most parts of the plant, is particularly well

seen in the oogonia and antheridia. Hartog, curiously enough, has partly verified and accepted these observations. The affinity between *Peronospora* and *Saprolegnia* is sufficiently close to justify us in viewing with extreme suspicion any such differences in the karyology as would involve nuclear divisions in the one genus and nuclear fusions in the other.

Although I am not prepared to accept Wager's results in detail, and, for a reason which will appear later, am much inclined to give them another interpretation, one must admit that the observations show inherent evidence of being thoroughly trustworthy: in particular the evidence as to nuclear division appears to me to be very strong. I have little doubt indeed that Wager's figures, together with my own, furnish considerable evidence for the occurrence of reducing divisions in *Peronospora* also.

The researches on reducing divisions carried out by Overton ('93), Strasburger ('94), and others, make it at any rate not improbable that such divisions should take place in the Saprolegnieae and Peronosporaeae.

In addition to this positive evidence in favour of the occurrence of divisions in the oogonia, it may be useful to bring forward what evidence exists as to the improbability of nuclear fusions taking place.

Oltmanns ('95) has shown that in the oogonia of *Vaucheria*, where nuclear fusions were supposed to take place, no such phenomenon occurs. A number of nuclei pass into the oosphere, but before the basal wall is formed they all pass out again with the exception of one, which becomes the nucleus of the oosphere, and later unites with the sperm-nucleus to form the nucleus of the zygote. I may add that it has been my good fortune to have seen some of the sections upon which Prof. Oltmann's conclusions were based.

So far as I have been able to ascertain, there is no thoroughly reliable evidence for the occurrence of the fusion of nuclei in the Thallophytes, except in connexion with the sexual process.

It must, moreover, not be forgotten that Dangeard came to

the conclusion that in the later stages of the development of the oogonium the substance of the nuclei becomes finely divided and distributed through the protoplasm. Such a view, while manifestly incorrect, might be formed naturally enough from a study of unsatisfactory preparations: my own earlier work caused me to form a somewhat similar opinion. It is difficult, however, to see in what way Dangeard could come to the conclusion indicated if the nuclei really undergo fusions.

I may also refer to Hartog's description of the nuclei at this critical stage of development of the oogonium, already given on p. 613. In particular, the fact that *the chromatin-masses are smaller* tells strongly in favour of *division*, and that '*they take up stain less readily*' appears to confirm the observations I have already made on *degeneration*.

Vacuoles in the Oogonium. The vacuoles in the oogonia have given rise to much discussion. In recent years they appear to have been confounded with the nuclei, as in former times they were confounded with the pits in the wall of the oogonium. They occur in the protoplasm of the oogonium from the time that the reducing division has led to the liberation of the paired rods up to the formation of the thin layer of protoplasm. They virtually exist then during the time that the degeneration of the nuclei is going on. They occur in their most typical form, as represented in Fig. 11 *a*, at the time of the completion of the division of the nuclei. The study of sections, moreover, shows that they are even more irregular and inconstant than would be suspected from a study of the fresh material. I believe that many of the so-called vacuoles observed in fresh material are simply thin places in the lining layer of protoplasm, as may be seen, for example, in Fig. 11 *b*. They have no morphological connexion with the nuclei, and with their possible physiological importance we cannot concern ourselves here. They cannot possibly be the means, as Hartog supposes, of bringing the fusion-nuclei into proximity with each other: this is well seen in Fig. 11 *a*, where the vacuoles are well developed, but the small nuclei are

equally distributed in the protoplasm, and show no tendency even to come together in pairs, much less to undergo fusion.

Development of the Antheridia up to the formation of the Fertilization-tubes. The antheridia have not been studied so thoroughly as the oogonia. The results won by the more difficult examination of the development of the oogonia, enable one to follow with ease the corresponding stages in the antheridia. Figs. 5, 9 and 10 show antheridia in successive stages of development. At the time of the separation of the antheridium from the antheridial branch by the formation of a basal wall, its protoplasm encloses a small but variable number of nuclei. These undergo division exactly as in the case of the nuclei of the oogonia. Fig. 8 *c* and *d* represent two nuclei from an antheridium. Degeneration of the nuclei certainly takes place in the antheridia at a very early stage, just as was seen to be the case in the oogonium. When the oospheres have been developed the antheridia give rise to the fertilization-tubes, and these apply themselves closely to the oospheres.

Some of the small nuclei pass into these fertilization-tubes and are there specially easy to see ; others remain behind in the antheridium and ultimately degenerate, as indeed do many of those which pass into the fertilization-tubes. This later degeneration has been observed by many botanists, and has long been thought to furnish evidence of the absence of fertilization.

Fertilization. The most detailed observations on fertilization were made on *S. dioica*, the species first studied, on sections stained with acetic-carmin. Early in the course of my investigations young oospores were found with two large nuclei, corresponding closely to those of *Achlya americana* figured by Humphrey. As the uninucleate character of the oospheres had already been placed beyond doubt, there remained but two alternatives to account for the presence of these two nuclei: they must have arisen either by the division of the pre-existing nucleus, or a new nucleus must have passed into the oosphere from some outside source. The structure

of the nucleus, too, at this stage, presented some difficulty, as it was distinctly unlike that of the oosphere-nucleus. A careful search was consequently made for stages intermediate between these two which would account for the change in the nuclei. These were soon found, and it was noticed that the nucleus of the oosphere gradually became larger and more irregular: indeed, the appearances led me at first to suppose that the chromosome-mass divided up into small portions which laid themselves in contact with the nuclear wall. Though I now believe this view to be incorrect, it will be seen from Fig. 17 that it was more or less reasonable. As a matter of fact, in consequence of the study of the dividing nuclei of the oogonium and a later examination of the oospore-nuclei in *S. Thureti*, I have come to the conclusion that the small gameto-nuclei undergo changes in virtue of which the microsomata in proximity to them become deeply stained; the chromosome-mass becomes of smaller and the nucleio-hyaloplasm of larger volume. As the small chromosome is more or less masked by the large microsomata, it is difficult to detect it in carmine-preparations at this stage. These changes in the nucleus agree in most respects with those which precede its division in the oogonium.

During the course of this investigation of the nuclei, oogonia in the condition represented in Fig. 18 were several times observed. I think they place beyond all doubt the occurrence of fertilization in *S. dioica*. The nuclei of the fertilization-tubes and oospheres are at this stage of identical structure. A nucleus is frequently seen at that point of the fertilization-tube which is in contact with the naked oosphere. Occasionally one observes, as in the figure, a second nucleus in the oosphere lying at its extreme edge, and in such cases one always finds by careful examination that a delicate cell-wall has been formed around the oosphere, and that there is a fertilization-tube exactly opposite to this lateral nucleus. No opening can be seen in the tube, and it would in any case be probably very difficult to prove its presence even were it there. But a permanent opening does not appear to be

necessary for fertilization to take place in such cases. No one, for example, has been able to determine the presence of a permanent opening at the end of the pollen-tube of the Angiosperms, where fertilization undoubtedly takes place. This remark has a special significance if we remember that Hartog has actually described the passage of Monads into the hyphae of Saprolegniaeae, and expressly states that the opening made is not a permanent one. It is well known too that in these plants little protoplasm escapes from the cut end of a hypha, the damage being repaired with extreme rapidity by the formation of a new piece of cell-wall, possibly owing to the presence of cellulose as a reserve material in a specially soluble form.

The young wall of the oospore is generally easily seen owing to the slight contraction of the protoplasm induced by the method of preparation, as is represented in the binucleate oospore of Fig. 18.

After a time, the two gameto-nuclei lie fairly closely together near the centre of the oospore. Fig. 19 represents the young oospores in this condition. Nuclei are still to be seen in the antheridia and fertilization-tubes. The two nuclei do not always appear in the same section, so that careful drawings and comparisons have to be made to convince oneself that the oospore is always binucleate at this stage. The oospores of two large oogonia were investigated in this way, and a large number of other oospores examined in a less rigorous manner. As the result of these observations, extending to hundreds of oospores, I was forced to the conclusion that the young oospores are *invariably binucleate*.

An examination of the nuclei in Fig. 19 shows the increase in size already described: the nuclei of the oospores are very much larger than the nuclei of the oospheres and fertilization-tubes.

The study of *S. Thureti* was undertaken to place the process of fertilization beyond doubt. As already pointed out, the absolute identification of the species, owing to the behaviour of the cultures, has here become impossible. Figs. 20

and 21, however, illustrate sections of oogonia in successive stages of development, which were almost certainly those of *S. Thureti*. They were certainly destitute of antheridia. The oospores are uninucleate, and exhibit those changes in the structure of the nucleus already described. The whole of the oospores in both of these oogonia, as well as a large number of others, were carefully studied, and *every oospore proved to be uninucleate*. A careful examination of the nucleus in Fig. 21 will lead to the conviction that it corresponds to De Bary's 'Kern-fleck,' and that Dangeard's view that it represents the first stage in the development of the fatty mass, must then fall to the ground.

I had anticipated that the application of the new and improved method of staining with gentian-violet and eosin to the study of *S. mixta* would yield valuable confirmatory results, but have been disappointed. At the period of fertilization the borders of the oosphere stain deeply with gentian-violet, and numerous small deeply stained granules appear in the protoplasm, so that the critical stages, as to which confirmation was needed, were more or less obscured. A return to the earlier method of staining with acetic carmine produced better preparations, but even these were not sufficiently good to give unquestionable results. When the young oospore has developed a little, and the nuclei in particular have increased in size, we find that *many of the oospores are binucleate*, as represented in Fig. 22, *while others are uninucleate*.

A long study of this perplexing condition, involving the careful examination of hundreds of oospores, has led me to the conclusion that the development in the uninucleate forms is apogamous, that is to say, no fertilization takes place; while in the binucleate forms fertilization occurs, and the nuclei are respectively male and female. This conclusion is based solely upon the absence of all evidence for either the fusion or division of the nuclei in the young oospore, and the important fact that the gameto-nuclei—as I must term them—may be found close together, though still distinct, in oospores much older than those which contain a single nucleus.

The oogonia may contain binucleate oospores alone, uninucleate oospores alone, or a mixture of these in various proportions.

In *S. mixta* then we conclude that fertilization frequently takes place, but in default of its occurrence the oosphere may develop parthenogenetically. The diclinous, androgynous, and completely apogamous forms investigated, thus form a very interesting series as regards sexual capacity: *S. dioica* is typically sexual, *S. Thureti* is typically apogamous, and *S. mixta* occupies an intermediate position.

An examination of Humphrey's figure of a binucleate oospore of *Achlya americana*, already referred to, leaves no room for doubt in my mind that a process of fertilization takes place in this species also. The figure of the uninucleate oospore—provided that the other nucleus has not been overlooked—leads me to believe that an apogamous development of the oosphere is also possible in this case. Humphrey's view that these constitute the last pair of fusion-nuclei is manifestly incorrect.

Hartog ('89), who appears to have investigated one or two other species of *Achlya* (either *A. recurva* or *A. prolifera*, or both), agrees with Humphrey that the last fusions of the nuclei may be delayed in *Achlya* until the formation of the young oospore. This remark leads me to believe that a second (if not a third) species of *Achlya* at least undergoes occasional fertilization. The fact that Hartog did not find binucleate oospores in *Saprolegnia* may be traced most probably to an examination of apogamous forms like *S. Thureti*.

It appears clear that not the least interesting of the results of this investigation is the discovery of a *distinct and unequivocal process of fertilization in at least four species from a group which has for many years been almost universally regarded as affording one of the most interesting cases of complete apogamy.*

Maturation of the oospore. This was studied first in *S. dioica* by means of preparations stained with acetic carmine or

haematoxylin. Later, during the examination of *S. Thureti* and *S. mixta*, an attempt was made to obtain better preparations by using gentian violet and eosin, but this proved entirely unsuccessful.

The difficulty of following the processes connected with the maturation of the oospore is extreme, not only because of the changes in the cell-wall of the oospore, which prevent the efficient action of the fixing reagents, but also by reason of the appearance in the ripening oospore of entirely new and puzzling structures. Fig. 23 *a, b, c, d* and *e* represent successive stages in the development of an oospore. The nuclei long remain distinct, but ultimately come together and lie closely side by side or end to end as represented in *b*. The histological details are difficult to follow, but it is easy, up to this point, to satisfy oneself of the indubitable nuclear character of the structures by a study of successive stages. In *e* is represented a uninucleate oospore, but as I have not been able to observe those stages which illustrate the actual fusion, I do not attach much value to it. At this stage it would be easy to overlook one of the nuclei under certain circumstances, as, for example, when side by side and in the same vertical plane.

In the stage represented at *b* the protoplasm is seen to contain deeply stained granules like large microsomata, lying in the knots of the protoplasmic meshwork. These appear to correspond to the drops of glycogen observed by Dangeard. They rapidly increase in size, as may be seen by comparing *b, c, d* and *e*. Ultimately they are seen, especially clearly in the haematoxylin preparations, to consist of hollow spheres, rings, or crescents. Finally, they increase further in size and diminish in number by fusing together in pairs until, at a late stage in the maturation of the oospore, they are reduced to a few rings or crescents, or sometimes a single hollow or more or less chambered sphere. That the rings are not optical sections of hollow spheres is proved by the fact that they frequently overlap each other, as well as by the appearances presented by optical sections from various points of view.

What are these curious structures? As they make their appearance coincidently with the fatty reserve material of the oospore, and the fusions observed amongst them appear to coincide with the fusion of the oily globules, I think it is very probable that they have some connexion with the production of this reserve material. They take up all nuclear stains that I have experimented with more readily even than the chromosomes, and all my efforts to discriminate between them and the nuclei by differentiating staining failed. The only test by which they can be distinguished is that of structure, and as the structure of the nuclei, owing to the incomplete fixation, is not well seen in nearly ripe oospores, there is a great difficulty in distinguishing between the nuclei and some of the forms assumed by these bodies. As I have not tested them microchemically in the fresh condition nor paid any attention to them except in stained sections, I must refrain for the present from expressing any definite opinion as to their character and significance. For some time I worked on the assumption that they were analogous to the pyrenoids seen in so many of the Algae. It is not unlikely that they simply form a special part of the fatty globules, in other words are a special form of reserve material. It seems very probable that it was these bodies which led Dangeard to regard the ripe oospores as multinucleate; this is all the more strange as he ('90 *b*) has described similar structures as occurring in the oospores of *Cystopus candidus*.

This study of the maturation of the oospores leaves the fusion of the gameto-nuclei undetermined, and it was mainly in consequence of this that I resolved, if possible, to follow the germination of the oospores.

Germination of the oospores. Specially good cultures of *S. Thureti* were obtained in September, 1894, and these were left in their jars for six months. In March, 1895, one of the cultures was removed to a warm room, the water changed and fresh flies added. The new flies were soon infected, and an examination of the old material then showed that many of the oospores were in all stages of germination. In germination,

as is well known, the wall of the oospore becomes thinner and more permeable to reagents, and the fatty mass disappears. A germ-tube is generally produced, and finally a club-shaped sporangium is formed. No case of the formation of a germ-tube was noticed in the fresh material, the swollen oospore itself becoming the sporangium. Moreover, the zoospores appeared, as a rule, to remain unliberated and to undergo germination inside the sporangium. This mode of development is not infrequent in the normal sporangia of old or somewhat unhealthy cultures.

The material thus obtained was sectioned, and very beautiful preparations were the result. While the wall of the oospore is still of considerable thickness, and the protoplasm still takes on that peculiar reddish tinge with the gentian-violet and eosin which I had already found to characterize the resting oospore, *the oospore contains one nucleus only.* This nucleus has the same structure—the typical vegetative structure—as those of the zoospores, mycelium and sporangia, as is shown in Fig. 24. In Fig. 25 we see a binucleate oospore with the nuclei still near to each other. The divisions appear to be in all cases direct. Figs. 26, 27 and 28 represent oospores with 5, 6 and 8 nuclei respectively. In these successive stages of development it will be noted that there is a gradual thinning of the wall of the oospore, an increase in its size, and the formation of a central vacuole and zoospore-rudiments, while the nuclei undergo division. Fig. 29 represents a section of an oospore which had produced 11 zoospores not yet entirely separated one from the other. Ultimately we find in the cavity of the oospore from 8 to 12 zoospores in various stages of germination. I have seen zoospores with from 1 to 5 nuclei and germ-tubes of various lengths. In the case of one oospore, represented in Fig. 30, which possessed 12 nuclei and was the only oospore in the oogonium, a germ-tube was produced, no doubt with a view to the formation of the typical club-shaped sporangium.

The uninucleate character of the oospore at the commencement of germination and its subsequent behaviour finally

remove all doubt as to the incorrectness of Dangeard's view, and makes it certain that the gameto-nuclei undergo fusion at a late stage in the maturation of the oospore.

Chlamydospores. The chlamydospores, as might be expected, contain nuclei of the normal vegetative type. (Fig. 31.)

GENERALIZATIONS AND CRITICISMS.

Generalizations based on the examination of a single genus must necessarily be of small value: indeed those which have been already made on apocytial plants, by at least one observer, have in this paper been proved to be quite valueless. It is obviously necessary that a large number of the genera of the Phycomycetes should receive a thorough investigation, and I have already made preparations for this purpose.

Some points of wider interest have, however, suggested themselves to me in consequence of the special study upon which I have been engaged.

The observations of Wager appear, as already stated, to be thoroughly reliable, but they are nevertheless manifestly incomplete. The nuclei in the gonidia, in particular, have a structure totally distinct from that figured for those of the mycelium and sexual organs. No indication is given as to how the one condition passes into the other. Wager's paper, however, deals mainly with so-called karyokinetic figures, and it may well be that the undividing nuclei have been more or less neglected.

In the early part of my investigation I met with some curious appearances in a few oogonia of *S. dioica*, a section of one of which is represented in Fig. 32, which may throw some light on this matter. In these sections I imagined I had found karyokinetic figures exactly similar to those described by Wager. Later on, towards the end of the research, and after the expenditure of much effort, I found the correct explanation of these appearances, and this may readily be guessed from the

examination of the antheridium in Fig. 10 *b*. The apparent karyokinetic figures are produced by the stained granules on the nuclear wall at the time the nucleus is undergoing division, and the dark-coloured spindle is the nucleo-hyaloplasm. The chromosome-rods, already half the size of the original mass, are frequently at this time only distinguishable with difficulty from the larger granules.

May not the figures of Wager be explained in the same way? If the resting nucleus has really a single chromosome, as the figure of the gonidium seems to indicate, this must be regarded as very probable; and should it be so, we might expect the karyology of the two genera to agree very closely. So much has this view appealed to me that I have already commenced an examination of *Cystopus candidus* with a view of putting it to the test, and have secured material for the study of *Peronospora parasitica*.

The evidence for the occurrence of fusions of nuclei in the unfertilized egg of *Peronospora* is extremely weak. Indeed, Wager's figures suggest to me that no such fusion occurs.

In the theory of heredity propounded so brilliantly by Weismann, the admixture of the substance which is the bearer of the hereditary tendencies during the sexual process is looked upon as the chief cause of variation in the higher organisms. An apogamous group, such as the Saprolegnieae was supposed to be, offers a special difficulty to the acceptance of this theory by botanists. The difficulty, however, is not a real one, as Weismann expressly rejects Nägeli's view that protoplasm has an inherent tendency to vary along certain definite lines of development, and, consequently, he is of necessity driven to adopt the view that it varies under the direct influence of the external conditions. The sexual process becomes specially important in those organisms in which the gametes are protected by their position from the direct action of the external conditions. Such apocytial plants as the Saprolegnieae, however, have their gametangia as much exposed to this direct action as the mycelium or zoospores, and the

nuclei of the sexual organs, it must be remembered, pass into them from the mycelial hyphae.

Although the difficulty presented by the Saprolegnieae is not a real one, it is of special interest to note that, even were this the case, the difficulty disappears under the light of the few observations already made on the functional character of the antheridia. Apogamy certainly must inevitably take place in the two species in which there is absolutely no provision for fertilization. In the other species, more or less closely related to *S. mixta*, in which apogamy *may* take place, antheridia are sometimes present; and after what has been said, we may be permitted to believe that fertilization *may* occur whenever antheridia and fertilization-tubes are present until this has been proved to be impossible. The Saprolegnieae as a group must therefore be no more regarded as apogamous than the Characeae, the Vascular Cryptogams, or the Phanerogams, in all of which isolated apogamous forms occur.

It might have been expected that the apogamous oogonia investigated would have differed from the others by their nuclei not undergoing a reducing division. Weismann, indeed, anticipated that this would be the case with the parthenogenetic ova of animals. The non-occurrence of the reducing division would then have accounted for the power of parthenogenetic development. As matters stand, we have to account for this by assuming—and there are excellent grounds for such an assumption—that the half-chromosome, if not increased to a whole-chromosome by the sexual process, can attain to the required mass by a process of growth during the resting period of the oospore. Since the oospheres and zoospores are generally admitted to be homologous organs, this is nearly equivalent to the statement that the oospheres simply revert to their primitive asexual condition.

The formation of azygospores in both algal and fungal conjugate forms, and the apogamous development of the old gametes of *Botrydium*, which appear to be well authenticated, to say nothing of the development of weak plants of *Ulothrix* from gametes which have not succeeded in conjugating, may be

mentioned as phenomena of which a similar explanation may, with great probability, be given. A thorough investigation of the nuclei of these plants is much to be desired.

The occurrence of fertilization in the androgynous forms is certainly very curious and puzzling. Nuclei, so closely related by descent as to be less than ten generations distant from each other and maintained throughout life under apparently identical external conditions, pass into the separate gametangia, and after undergoing a reducing division fuse with one another to form the nucleus of the zygote. This phenomenon seems to involve the assumption that the nuclear divisions are qualitative as well as quantitative in character. If this be so, the advantage of the sexual process in promoting variability is evident.

I should like to make clear that there is nothing exceptional in the degeneration of nuclei as described in these plants, and any peculiarity in the details of the phenomena are readily explained as special adaptations. The majority of gametes, in the animal as well as in the vegetable kingdom, die, and are a serious loss to the organism, or rather to the species. The gameto-nuclei in the oogonia of *Saprolegnia* which are present in excess of the required number evidently die, but they are not a great loss to the plant, as their substance is almost certainly utilized by the oogonium at once as food, or is stored up as reserve material for future use. The protoplasm, which we may regard as having belonged to the defunct nuclei, is not lost at all, but goes to increase the size of the oosphere—probably a decided advantage to the plant. Thus it happens that when the nucleus of the zygote divides after its period of rest, the number of nuclei is restored, and the requisite protoplasm to furnish each with a sufficiency for spore formation is already present. In consequence of this, about as many zoospores are produced by the oospores of an oogonium as there were nuclei enclosed in it at the time of the formation of its basal wall.

I cannot bring these remarks to a close without recording my conviction that Strasburger's ('94) interpretation of the

periodic reduction of the chromosomes does not adequately explain the facts. So far as concerns the Thallophytes too, it may be safely asserted that his prophecies have not been fulfilled. He asserted, and at the time with sufficient justification, that 'the assumption that this reduction takes place during the development of the sexual organs is not supported by any direct evidence,' and then proceeds to suggest that the necessary reduction takes place in the lower Cryptogams during the germination of the zygote. The facts already mentioned show quite clearly that a reducing division takes place in *Saprolegnia*, in the gametangia and not in the zygote.

The great objection which all biologists must raise against Strasburger's view is that it necessitates one explanation of the reducing division in plants and another in animals without sufficient reason. *Saprolegnia* and most other Thallophytes (like animals) have no sporophyte-generation. If the real significance of the reducing divisions observed in higher plants is simply the return of the sporophyte-cells to their primitive gametophyte-condition, what meaning are we to attach to the corresponding process in *Saprolegnia* and animals where no such return is possible? It would needlessly complicate matters to assume that the zygote and its products constitute a sporophyte-generation in *Saprolegnia*, for they certainly do not; and even if they did, no reducing division takes place in it.

In plants in which there is no sporophyte-generation it would be reasonable to admit that the reducing division occurs, as in animals, in the sexual organs; and that the number of chromosomes is restored by the sexual act. The phenomenon consists essentially of a halving and a subsequent restoration to the whole condition, and this we have proved to take place in a characteristic manner in *Saprolegnia*.

In plants in which there is a distinct sporophyte-generation there really appears to be no halving in connexion with the sexual process, but rather a doubling, and a reduction to the undoubled condition takes place in the transition from sporophyte to gametophyte. When we consider that the sporophyte

is a new generation intercalated between two successive gametophytes, and that it is entirely unrepresented amongst animals, it is not unlikely that the reducing division has in this case a special significance.

In fact, I would suggest that the evolution of the sporophyte was initiated by the fusion of gametes in which the halving observed in animals and Thallophytes did not take place; and that the increase in the mass of the nucleus had the effect of a stimulus in the direction of spore-formation, giving to the organism the advantages of polyembryony. The power of subsequent return to the undoubled condition must, on this theory, have been developed simultaneously with the sexual capacity of the abnormal gametes. Wallace has conclusively shown that such simultaneous variations certainly occur.

Such a view is, at any rate, supported by the facts already ascertained, and will certainly have the effect of stimulating me, and possibly others, to further research. An investigation of the karyology of *Oedogonium*, *Coleochaete*, and some genera of the Florideae seems particularly desirable.

The results of Klebahn's investigation of *Closterium* and *Cosmarium*, and Chmielewsky's investigation of *Spirogyra*, appear to require confirmation. The facts seem to me to support the theory propounded here. The whole course of development and the minutest details as to the structure of the nuclei must, however, be determined before the significance of the behaviour of the nuclei can be made clear.

SUMMARY.

1. The nucleus in the genus *Saprolegnia* is bounded by a nuclear wall, and possesses one central chromosome of spongy texture. The space between the nuclear wall and chromosome is occupied by a nucleo-hyaloplasm, which is traversed by fine threads.

2. The nucleus undergoes direct division in the zoospore

and mycelium, and the products of these divisions ultimately pass into the sporangia and gametangia.

3. Neither nuclear divisions nor nuclear fusions take place in the sporangia.

4. In the oogonia and antheridia each nucleus undergoes one reducing division by an indirect method, in virtue of which the whole chromosome becomes a half-chromosome, but no fusions of functional nuclei take place.

5. The number of gameto-nuclei produced in the oogonium by the reducing division is about twenty times greater than that necessary to provide one nucleus for each oosphere. The number is reduced by the degeneration of the excess.

6. Most of the gameto-nuclei in the antheridia and fertilization-tubes also undergo degeneration.

7. Fertilization takes place invariably in *S. dioica*, and at least occasionally in *S. mixta*, *Achlya americana*, and another species of *Achlya*, while *S. Thureti* is normally apogamous. The whole-chromosome condition is restored to the nucleus either by the sexual process or by a process of growth. The two gameto-nuclei do not fuse to form the single zygote nucleus until a late stage in the maturation of the oospore. The Saprolegnieae as a group is not apogamous.

8. At the period of germination of the oospore the nucleus of the zygote undergoes direct division to furnish one nucleus for each zoospore.

9. The sporophyte-generation of the higher plants probably owes its origin to the fusion of gametes whose nuclei did not undergo a reducing division. The doubling of the chromosomes acted as a stimulus to spore-formation, and involved a halving of the chromosomes in the return to the undoubled condition of the nuclei in the gametophyte-generation.

UNIVERSITY COLLEGE, CARDIFF,

September, 1895.

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EXPLANATION OF FIGURES IN PLATES XXIV AND XXV.

Illustrating Mr. Trow's paper on the Karyology of *Saprolegnia*.

The outlines of all important structures in the figures were traced with the camera lucida and the details then filled in by freehand. The highly magnified figures of the separate nuclei were, however, drawn entirely by freehand. Those referring to *S. dioica* were drawn from preparations stained with Schneider's acetic carmine and with the aid of Zeiss's apochromatic 2 mm. objective (aperture 1.3) and the compensating ocular 4. The magnifications given were determined by measurement.

The other figures were drawn from preparations stained with gentian-violet and eosin and with the aid of Zeiss's $\frac{1}{2}$ achromatic homogeneous immersion objective (aperture 1.3) and the Huyghenian oculars 2 and 4.

All the nuclei in a section have as a rule been represented by reducing them to the same optical section. Sections in which there were nuclei overlying one another were rejected as unsuitable.

Abbreviations: *an.* antheridium; *ch.* chromosome; *c. w.* cell-wall; *deg. n.* degenerate nucleus; *d. n.* dividing nucleus; *f. g. n.* female gameto-nucleus; *g. n.* gameto-nucleus; *m. g. n.* male gameto-nucleus; *n.* nucleus; *n. m.* nuclear membrane; *nu. hy.* nucleio-hyaloplasm; *oog.* oogonium; *v.* vacuole.

S. mixta, De Bary.

Fig. 1. *a.* Resting zoospore showing the structure of the nucleus. The deeply stained granules between the nucleus and the vacuole disappear before germination. *b, c,* and *d.* Germinating zoospores showing the division of the nucleus, and the formation of the germ-tube. The nuclei in *d* were further apart than they appear to be, the close grouping being partly due to the necessary process of reducing them all to the same optical section. $\times 1150$.

Fig. 2. *a* and *b.* Hyphae: *a* is a tangential section, *b* has been sectioned at various angles. The nuclei in *b* are abnormally elongated. $\times 1150$.

Fig. 3. *a.* Transverse section of a nearly mature sporangium. The arrows A and B point in the direction of two zoospore-rudiments whose nuclei were respectively above and below the optical section figured. $\times 1290$.

b and *c*. Radial and tangential sections respectively of the lower portion of a younger sporangium. The strands of protoplasm connecting the young spores are well seen. $\times 1150$.

Fig. 4. Median section, $7.5\ \mu$ thick, of a young oogonium which contained 72 nuclei. Its diameter was $29\ \mu$. $\times 1150$.

Fig. 5. Median section of an oogonium. The basal wall has been formed. Three of the antheridia attached to it are also represented. The nuclei are preparing for the reducing division. $\times 660$.

Fig. 6. *a, b, c, d*, and *e*. Nuclei undergoing the reducing division. In *d* the half chromosomes are seen in plan and in *e* in profile. The early stages are represented in *a* and *b*. The nuclear wall appears to be breaking down in *e* and the nucleo-hyaloplasm is dispersing itself in the surrounding protoplasm. $\times 2300$.

Fig. 7. An oblique section of the oogonium in which the nuclei of Fig. 6 were found. The antheridia were not drawn. The two half chromosomes are seen in most of the nuclei. The intact nuclear wall appears still to prevent the escape of the nucleo-hyaloplasm into the surrounding protoplasm. $\times 1150$.

Fig. 8. *a* and *b*. Dividing nuclei from an oogonium. *c* and *d*. Dividing nuclei from an antheridium. The half chromosomes lie more or less free in the protoplasm. $\times 2300$.

Fig. 9. Median section of an oogonium, one of the antheridia attached to which is also drawn. The nuclei are in the condition represented in Fig. 8, and the stained masses of nucleo-hyaloplasm and microsomes are still evident. $\times 1150$.

Fig. 10. *a* and *b*. Two sections of one oogonium and sections of two antheridia showing the completion of the development of the gameto-nuclei (the end of the reducing division) and the commencement of the degeneration of the rejected gameto-nuclei. $\times 1150$.

Fig. 11. *a* and *b*. Tangential and median sections of an oogonium (antheridia not drawn) which had a diameter of $50\ \mu$ and contained 46 gameto-nuclei. The oogonium was in the typically vacuolated condition. $\times 1150$.

Fig. 12. *a* and *b*. Tangential and nearly median sections of an oogonium (antheridia not drawn) at the stage preceding the commencement of the formation of the oosphere rudiments. $\times 1150$.

Fig. 13. Three successive sections of an oogonium (antheridia not drawn) with three oosphere-rudiments in the earliest stage of development. Degenerate fusion-nuclei are to be noted in these, but only three typical gameto-nuclei, one near the centre of each rudiment. $\times 1150$.

Fig. 14. Section of an oogonium with one uninucleate oosphere-rudiment. The three remaining sections contained three additional uninucleate rudiments. $\times 1150$.

Fig. 15. Section of an oogonium with 4 nearly mature uninucleate oospheres. The antheridium is proceeding to develop a fertilization-tube. $\times 1100$.

S. dioica, De Bary. Fertilization.

Fig. 16. Section of a large oogonium (antheridia not drawn) with three uninucleate oosphere-rudiments. All the other rudiments in the oogonium were likewise uninucleate. $\times 610$.

Fig. 17. Median section of a large oogonium with mature oospheres. The spongy character of the protoplasm is beautifully seen and also the single central

nucleus. One antheridium is drawn. Its fertilization-tube which is already almost in contact with an oosphere contains two male gameto-nuclei. $\times 610$.

Fig. 18. Section of an oogonium and two antheridia showing fully developed fertilization-tubes and two newly formed oospores. $\times 610$.

Fig. 19. Section of an older oogonium showing the pair of gameto-nuclei in the oospores still unfused but decidedly larger. $\times 610$.

S. Thureti, De Bary. Typical apogamy.

(Possibly apogamous oogonia of *S. mixta*, De Bary.)

Fig. 20. Section of an oogonium with uninucleate oospores. The nuclei are still small. $\times 660$.

Fig. 21. Section of an older oogonium with uninucleate oospores. The nuclei have increased greatly in size and have the same appearance as those about to undergo the reducing division except that the chromosome is much smaller. $\times 660$.

S. mixta, De Bary. (Intermediate condition with respect to apogamy.)

Fig. 22. Section of an oogonium with oospores. One oospore is distinctly binucleate. The one to the right beneath had a second nucleus in another section. All the other oospores in the oogonium had certainly one nucleus, and some of them might possibly have had two. $\times 660$.

S. dioica, De Bary. Maturation of the oospores.

Fig. 23. *a, b, c, d,* and *e.* Oospores in successive stages of development. The large deeply stained granules around the nucleus in *e* are developed from the smaller ones seen in *b*. $\times 610$.

S. Thureti, De Bary. Germination of the oospores.

Fig. 24. Two uninucleate oospores. $\times 1150$.

Fig. 25. A uninucleate and binucleate oospore. The separation of the nuclei in the latter is just completed. The indentation in the wall is the result of the method of preparation and is rather exceptional. $\times 1150$.

Fig. 26. *a* and *b.* Successive sections of an oospore with 5 nuclei. $\times 1150$.

Fig. 27. *a* and *b.* Successive sections of an oospore with 6 nuclei. $\times 1150$.

Fig. 28. *a, b, c,* and *d.* Successive sections of an oospore with 8 nuclei, in which the zoospore-rudiments have already commenced to form. $\times 1150$.

Fig. 29. One section out of four, of an oospore in which eleven zoospores had just been formed. $\times 1150$.

Fig. 30. Section of a germinating oospore which contained altogether 12 nuclei, and has developed a germ-tube with a view to the formation of a normal sporangium. $\times 1150$.

Fig. 31. Tangential section of a chlamydospore of *S. mixta*. $\times 1150$.

Fig. 32. Tangential section of an oogonium of *S. dioica* showing pseudo-karyokinetic figures. The figures are due to imperfect preparations of nuclei in the process of undergoing the reducing divisions. $\times 610$.

A.H. Trow del.

A.H.Trow del.

Fig. 17.

Fig. 18.

Fig. 23.

Fig. 28.

29.

Fig. 30.

Fig. 31.

Fig. 32.

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