5. Studies on Opalina.

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In the Spring of 1900 Mr. Ernst Teichmann, at Prof. Boveri's suggestion, began a study of the cytology and reproduction of *Opalina caudata*. The study, however, was never completed, though several interesting results had been obtained. When I reached Würzburg, last fall, Prof. Boveri called my attention to the large size and clearness of the nucleus in this species and suggested that I study it. For this suggestion and for constant advice during the course of the work I am very grateful to him.

Most of Mr. Teichmann's preparations are mislaid and cannot be found. Some of his sections, however, I have used, and lately I have seen his drawings.

The study of *Opalina* has proven of much interest. The minute anatomy, especially of the nucleus, has been studied chiefly in *Opalina intestinalis* and *O. cautata*, each of which has but one pair of nuclei. Comparisons have been made with *O. ranarum*, *O. dimidiata*, *O. obtrigona* and with a new species which I will name *O. zelleri*¹. Excretory organs have been found in the two binucleate species in *O. obtrigona*, *O. dimidiata* and doubtfully also in *O. zelleri*. These have been described in a paper now in press (Arch. f. Protistenkde.). The processes of reproduction, which occur in the spring, have been followed in the two binucleated species and in *O. dimidiata*. Infection experiments have been made with all the species named except *O. ranarum* and *O. zelleri*. I will report very briefly upon some of the results of this study.

Mitosis.

Opalina intestinalis.

The nuclear membrane never disappears during division. But remains intact, becoming dumbbell-shaped and finally separating into two parts, one for each daughter nucleus. These are connected for a long time by a slender thread, the attenuated membrane. The nuclear membrane is very firm. In nuclei which have been removed from the cell

¹ This in the species which Zeller found occurring with *O. dimidiata* in *Rana* esculenta. He thought it very likely a new species, but did not name it. To his description, which is sufficient for diagnosis, I will add a few points in a later publication.

and are lying in salt solution the membrane may remain intact for more than three days. The toughness and firmness of this nuclear membrane is of interest in connection with the question of the extrusion into the cytoplasm of certain chromatin masses is the nucleus, to be described a few paragraphs below.

A very delicate linin net with nodal granules is present, resembling exactly in structure and staining reactions the cytoplasmic net and granules. The real structure is that of a foam, at least in the "resting" nucleus.

The chromatin net in the *resting* nucleus consists of large and small chromatin masses and their branching anastomosing pseudopodialike processes. In certain conditions of the nucleus no such processes are found.

As division approaches, the longitudinal fibres of the linin net become emphasized, the transverse fibrils becoming less numerous and fainter. These longitudinal fibrils do not form a true spindle or any very marked or definite structure. The chromosome branches which run longitudinally are likewise seen to be thickened, while the lateral branches are drawn in or become much fainter. In nuclei stained with safranin and light green the distinction between these two kinds of fibrils (linin and chromatin fibrils) is very sharp and clear, the former being green and the latter red.

There is no clear equatorial plate of chromosomes at any stage, but one sees irregularly dumb-bell-shaped masses of chromatin (chromosomes) at the equator, just inside the nuclear membrane. These soon completely divide, except for connecting threads of chromatin, and the halves migrate to the two poles of the nucleus. As the daughter chromosomes pass toward the poles each is seen to be united to the pole by a thread of chromatin which grows thicker as it shortens during this migration.

No centrosomes are present inside or outside the nucleus, nor is there anything of special note at the poles of the nucleus. Achromatic granules are usually present here as they are throughout the nucleus, but there is generally no special aggregetion of them.

I have found no longitudinal division of the chromosomes, though Teichmann so interpreted and figured some of his preparations. The chromosomes are never compact and regular, but always remain more or less branched. They are best seen during the migration stages of mitosis and are then found to be eight in number (fig. 1). They differ in size and shape and in the number of chromatin granules they contain and these differences may be constant. The chromosomes at all times lie just inside the nuclear membrane, never near the center of the nucleus.

As the daughter nuclei are formed the chromosomes send out thin,

broad, bands-haped pseudopodia by which they become united. At this time they form a more or less compact mass. This passes later into the resting condition as described.

The chromosomes are at all times granular, though during the migration stages of mitosis these granules are usually less readily distinguished from the intermediate substance of the chromosomes.

During and following the compact stage the chromosomes become sharply differentiated into granular portions and homogeneous spheres. The chromatin spheres vary in size and number in different nuclei and in different chromosomes. The granular portion consists of a weakly-staining matrix (I believe usually in the form of a thin plate) containing darkly staining granules of slightly different sizes, the smallest being the size of the ordinary granules at the nodes of the linin net. The chromatin spheres disappear (at least as such) from the nucleus. Perhaps they pass into the cytoplasm through the ends of the nucleus, at the points where the nuclear membrane broke in a previous division. (Compare the description of the cytoplasmic spherules, given later.) At no stage seen do the chromosomes entirely disintegrate into granules. The plates of faintly staining chromatin uniting them into groups can always be discerned even in the most diffuse stages. It is not well here to attempt any discussion of the significance of the chromatin spherules.

The nucleolus, a true plasmosome, has not as yet been carefully studied.

The striated condition of the dividing nucleus passes into the net and foam structure of the »resting« nucleus by the restrengthening and new formation of lateral branches of the linin and chromatin fibrils.

Opalina caudata.

This species closely resembles *O. intestinalis*, except that it has six instead of eight chromosomes.

All the phenomena so far discribed, except those of the linin net and the lateral branches of the chromatin fibrils, have been seen in the living nuclei and cannot be artifacts.

The much smaller size of the nuclei and the greater number of the chromosomes in *O. ranarum*, *O. dimidiata*, *O. obtrigona* and *O. zelleri* render these species much less favorable for study.

Cytoplasmic spherules.

The cytoplasm of *Opalina* is divided into ectosarc and endosarc. Both contain in the vacuoles of their foam-like plasma very numerous spheroidal or more usually ovoid bodies (fig. 4). I believe that all individuals at all times of year contain these and that in cell division they are handed

down to the daughter cells, as are, for example, the pyrinoids of plant cells. The spherules of the endosarc resemble in size and form the larger chromatin spheres of the nuclei and they react to the same stains, but more faintly. It is possible they are derived from the latter, but I am not yet convinced that this is true. The endosarc spherules are highly refractive in the living animal. They do not stain intra-vitam with any of the usual intra-vitam dyes except neutral red, methyl violet, dahlia and gentian violet. One often finds the spherules of the endosarc showing a dumb-bell form and it is probable that they divide by constriction. Both in the living animal and in stained preparations they are seen at times to be granular, the more evident granules being peripheral while the core is apparently more nearly homogeneous.

The spherules of the ectosarc are larger, they stain with plasma dyes rather than chromatin dyes and take intra-vitam stains more strongly. In the living *O. intestinalis*, *O. caudata* and *O. dimidiata*, especially in the minute forms found in the spring, they are sometimes yellow. This yellow color is emphasized by acetic-carmine while the endosarc spherules are stained red.

During the whole year *Opalina* divides by fission. This is usually longitudinal, but occasionally transverse division is found. The latter is rare in the binucleate forms. During summer, fall and winter these divisions take place very slowly, days being required for the completion of one division. In the spring, as the breeding season of the host approaches, the rate of division increases, and the animals become very minute. They then encyst and pass with the foeces of the host into the water, further development taking place in the rectum of the tadpole. It is not necessary for the cysts to lie in water any time in order to secure good infection.

Conjugation.

Opalina intestinalis and O. caudata.

The infection cysts of these binucleated species are usually uninucleated though frequently one finds binucleated cysts. The animals which emerge from the cysts in the small intestine or rectum of the tadpole begin to divide by longitudinal and transverse division, the divisions being rather slow, apparently occupying at the least about one day. After from forty to eighty hours minute tailed gametes are found (fig. 2). Their tails are very sticky. Usually they swim with the tail in front. The tailed gametes are of two sizes, one about twice as large as the other, the smaller being formed from the latter by longitudinal division. I have never seen two tailed gametes conjugate as Neresheimer has described. Conjugation occurs between tailed gametes (larger or smaller) and non-tailed forms always somewhat and usually much, larger than the tailed form (fig. 3). This has been observed many dozens of times for both species. The tailed form at the time of conjugation has a single nucleus. The larger, non-tailed gamete may have one large "resting" nucleus, one large nucleus in process of division (fig. 4), or two nuclei,

Fig. 1.

Fig. 1. An adult but small *Opalina intestinalis* with each nucleus in mitosis, eight chromosomes in each end of cach nucleus. From an acetic-carmine preparation in

which the cilia were too much injured to be drawn. \times 620 diameters. Fig. 2. A microgamete of *Opalina intestinalis*, from life. The clear vesicle is the nucleus whose chromatin structures did not show clearly enough to draw. Observe the spherules in the endosarc; also the slight swelling near the end of the tail.

 \times 930 diameters.

Fig. 3. Conjugation in *Opalina intestinalis*, early stage. From life. \times 620 diameters.

the posterior one often being smaller. In conjugation the fusion is usually complete, though in several cases I have seen gametes more than

Fig. 2.

Fig. 3.

half fused finally separate, one, or both, or neither immediately encysting by throwing of the cilia and generally part of the protoplasm and forming a very delicate cyst (fig. 7). In a few cases encystment of the same sort followed complete fusion. It is not usual and I beleive that when it occurs it is abnormal. (Nere sheimer describes encystment as normal.) Observation of the nuclear phenomena of conjugation is still continuing. When the macrogamete has one nucleus it fuses obliquely, end to end with the nucleus received from the microgamete, I have once in the living animals seen the nucleus of the microgamete enter and fuse with the posterior nucleus of a binucleated macrogamete, and I have had acetic carmine preparations which indicate the fusion of the nucleus

Fig. 4.



Fig. 4. Conjugation in *Opalina caudata*. Fusion of the gametes has gone a little further than in the case shown in fig. 3. Observe the three chromosomes in each nucleus, also the spherules in the endosarc and ectosarc. Near the nucleus of the microgamete is a vacuole which in the drawing appears like a spherule of the ectosarc. From an acetic-carmine preparation. The cilia and the spherules of the ectosarc were omitted in the original sketch and have been supplied by comparison with other preparations. \times 620 diameters.

Fig. 5. A zygote of *Opalina intestinalis* in which the nuclei from the macrogamete are both in a late stage of mitosis, while the nucleus from the microgamete is not yet in division. The rays at the ends of the spindle-shaped microgamete nucleus are merely threads of the general cytoplasmic reticulum. Their resemblance to astral rays is deceptive. The body of the zygote is beginning to divide. From an acetic acid preparation in which the cilia were injured. \times 620 diameters.

Fig. 6. A macrogamete of *Opalina intestinalis* with two nuclei each containing four masses of chromatin, indicating the probable presence of four chromosomes in each nucleus. Nuclei of macrogametes of this species when in mitosis show clearly four chromosomes. From an acetic-carmine preparation. × 620 diameters.

Fig. 7. A macrogamete of *Opalina intestinalis* with the nucleus in mitosis (four daughter chromosomes in each end). This gamete was found in an early stage of conjugation (cf. fig. 3). Later it separated from the microgamete, which at once threw off its cilia and became spherical. Ten minutes later the macrogamete also became spherical and threw off its cilia and part of its cytoplasm, as shown in the figure. This pseudoencystment I think is pathologic. × 620 diameters.

of the microgamete with the anterior nucleus of the binucleated macrogamete. The phenomena immediately following conjugation have not yet been fully determined. They are too various for brief description (fig. 5).

The tailed gametes often, possibly always, arise by differential division, one product of the division acquiring a tail, the other not. The tailed gamete may itself begin to divide again by longitudinal fission before it has completely separated from its sister. I do not know how many divisions occur after an animal leaves its infection cyst before the gametes arise. The size of the infection cyst and of the animals that hatch from them varies so much that measurements of the minute forms cannot settle this question, and I do not succeed in rearing the isolated animals long enough to answer it.

The chromosome number is four for the microgametes and the macrogametes of *O. intestinalis* (fig. 6 and 7). It is three for the gametes of *O. caudata* (fig. 4). These chromosomes, as seen in the living animals, show about half as many granules as do the chromosomes of the full grown individuals. I hope by the study of sections to determine when and how this reduction occurs. It seems clearly to take place before the formation of the infection cysts. Neresheimer finds twelve chromosomes in the adult *O. ranarum* and twenty-four in the young spring forms before encystment, a result I do not understand.

Opalina dimidiata.

The infection cysts contain from two to seven nuclei, generally from three to five, most commonly four. The animals hatch from the cysts apparently with the same number of nuclei they had on entering the cyst. Both longitudinal and transverse division occurs after hatching. Tailed gametes of two sizes are formed as in the binucleated Opalinae. These I have never seen conjugate with one another. Of the conjugating gametes one was always found to be tailed and the other without a tail and usually very much larger. Normal encystment after conjugation was not found in this species, nor have I seen fusion of the nuclei in the zygote. The number of chromosomes in the gametes or the adults has not yet been determined. It is much less in the gametes, apparently half of the number of the adults. The tailed gametes always have one nucleus. The tailess macrogamete may have one or two.

At about the time the infection cysts are formed, peculiar phenomena are observable in the nuclei of *O. intestinalis*, *O. caudata* and *O. dimidiata*, which I will not attempt to describes in this condensed paper. They seem to be associated with a reduction in the amount of the functional chromatin and somewhat resemble the phenomena attending degeneration of the nuclei in abnormal *O. obtrigona*. The most noticeable

feature of these phenomena is the presence of one or two refractive and deeply staining disc-shaped masses in the nuclei. I find either one or two of these masses in the nuclei of the minute animals before encystment, one or two in the nuclei of the cysts in the frog's rectum, one or two in the nuclei of the cysts in the water, one or two in the nuclei of the cysts in the tadpole, one or two in the nuclei of the little Opalinae after hatching, and I have sometimes found what appear to be similar bodies in the nuclei of gametes during conjugation. If these masses are extruded into the cytoplasm, and some of my preparations seem to indicate that they are, the time of their extrusion seems to vary within wide limits. When two nuclei are present in the cysts or minute forms of any of these species, either nucleus may have none, one or two of these masses. When more than two nuclei are present (O. dimidiata), any of these nuclei may have none, one or two of the chromatin masses. It is seen that I do not find the phenomena quite so definite as Neresheimer describes. I have not yet, however, studied sections of these forms.

No complete degeneration of nuclei occurs in the binucleated Opalinae in connection with the spring reproduction, at least before conjugation, and I have seen nothing in any species to indicate that new nuclei are formed from chromatin masses extruded from the old nuclei into the cytoplasm, as Neresheimer describes. The nearest approach to this I have seen is the appearance of the cytoplasmic spherules with their peripheral granules, but these appearances are found throughout the year and are not connected with the formation of new nuclei. In certain abnormal Opalinae obtrigonae I have found many of the nuclei degenerating. Some of my preparations of *O. dimidiata* give some indication of a somewhat similar degeneration of some of the nuclei before and during the spring reproduction, but I have found nothing to suggest that many of the nuclei degenerate. I am not at all sure that any do. The question must be carefully studied upon sections.

Infection experiments.

Under natural conditions the several species of *Opalina* are found only in certain definite hosts. The hosts and parasites I have studied are related as indicated in the following table:

Host.	Parasites. <i>Opalina dimidiata</i>		
Rana esculenta			
		-	zelleri
Rana fusca	~	~	ranarum
Hyla arborea			obtrigona

Host.	Parasites.		
Bufo vulgaris	Opalina ranarum		
– variubilis }	- • dimidiata		
Bombinator igneus	- canduta		
- nachumus	- intestinalis		

I find by experiment that tadpoles of *Rana esculenta*, *Bufo vulgaris* and *Bombinator pachypus* may each be readily infected with any or with any two or with all three of the species *Opalina intestinalis*, *O. caudata*, *O. dimidiata*, by feeding the cysts to the tadpoles. I have not experimented with the cysts of *O. ranarum*, *O. obtrigona* or *O. zelleri*. Young and full-grown frogs of *Rana esculenta* are easily infected from cysts of *O. intestinalis* (I have not tried cysts of other species): young toads (*Bufo vulgaris*) can be infected from cysts of *O. intestinalis* and *O. caulata* (I have not tried cysts of other species), also adult *Hyla arborea* can be infected from cysts and from adults of *O. intestinalis*.

Adult Opalinae, fed to tadpoles, are in part digested, but many pass through the intestines unharmed and establish apparently thriving colonies in the recta. I have infected tadpoles of *Rana esculenta*, *Bufo vulgaris* and *Bombinator pachypus* each with adults of each of the following species *Opalina dimidiata*, *O. obtrigona*, *O. caudata* and *O. intestinalis*. It is probably true that tadpoles of any species can be infected with either cysts or adults of any species of *Opalina*. I cannot yet say whether the Opalinae thus introduced to an unnatural host will continue to live longer than four weeks, but they grow and thrive for that length of time and probably live indefinitely. It seems probable that such cross infections occur frequently under natural conditions. Why, then, do we find the distribution of the several species of *Opalina* in the different frogs and toads so restricted? The cysts introduced into an unnatural host hatch, the little animals which emerge dividing and forming gametes which conjugate.

For our knowledge of *Opalina* we are indebted chiefly to Zeller's² beautiful paper and to Neresheimer's³ very interesting preliminary note. I have made no attempt to discuss either of these, or any other, papers, referring only to a few disenpancies between my work and Neresheimer's.

Würzburg, July 1 st 1907.

² Želler, E., Untersuchungen über die Fortpflanzung und die Entwicklung der in unsern Batrachiern sehmarotzenden Opalinen. Zeitschr. f. wiss. Zool. Bd. XXIX. 1877. p. 352-379.

³ Neresheimer, E., Der Zeugungskreis von *Opalina*. Sitzgsber. d. Ges. f. Morph. u. Phys. in München, 1906. Hft 1. Read July 15th 1906.

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