Uroleptus Halseyi Calkins.

11. The origin and fate of the macronuclear chromatin.

Von

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(With 1 figure in the text and plate 11—12.)

We have very little exact knowledge of the functions of the ciliate macronucleus or of the changes which go on at different periods of cell activity. Popoff (1907) indeed has made a careful study of the volumetric relations in *Frontonia leucas* and other ciliates at different periods of cell life but he was not concerned with the changes in the finer structure or make-up of the macronucleus. It is generally assumed (e. g. Doflein-Reichenow 5th Ed, p. 29) that the macronucleus of the ciliates is somatic in character while the micronucleus is generative and this assumption is borne out by the continued life of amicronucleate races. For example *Oxytricha fallax* without micronuclei was cultivated by Woodruff (1921) through 246 generations and *Urostyla grandis* through 158 generations: *Oxytricha hymenostoma* by Dawson (1919) through 289 generations. Other amicronucleate races have been cultivated for varying periods by other observers (Patten 1921 *Didinium nasutum*; Landis 1920 *Paramecium caudatum*; Moody 1912 *Spathidium spathula* etc.). While in some cases on record we have legitimate grounds for doubting the correctness of the observations, there is no doubt in the majority of cases, and it may be safely stated that it is possible for some ciliates to live and to divide in the absence of a micronucleus. This however appears to be the limit of acti-

vities for in no amicronucleate race has a new micronucleus been derived from a macronucleus and in no case has fruitful conjugation occurred. Such amicronucleate races are apparently due to faulty reorganization after conjugation or possibly to the exclusion of a daughter micronucleus upon division of the cell. PRANDTL (1906) for example, describes the failure of some exconjugants of *Didinium nasutum* to form a micronucleus, both halves of the divided zygote nucleus forming macronuclei. In normal cases throughout the entire class Ciliata the zygote nucleus or amphinucleus gives rise to products one of which becomes differentiated as a macronucleus another as a micronucleus. It is somewhat misleading therefore to state that after conjugation the new macronucleus comes from the micronucleus — it does not — it comes from the amphinucleus before micronuclei are differentiated as such.

Whatever may be the origin of amicronucleate races the fact that a ciliate may continue to carry on metabolic activities and to live without a micronucleus, must I believe, be accepted. But it is also a fact, determined by repeated experiments, that an enucleated fragment of a cell cannot carry on constructive metabolic activities and therefore cannot live more than a few hours, if at all. The nucleus then is an essential factor in cell life and its functions appear to be centered in the constructive phases of metabolism. An enucleated fragment may move as usual and may take in solid food substances but it cannot digest or assimilate such food, hence it would seem that the digestive ferments are associated in some way with the nucleus. In amicronucleate races it would be with the macronucleus, and in all probability it would be with the macronucleus in all ciliates.

It is worth while therefore to study the finer structure of the macronucleus both morphologically and so far as possible, chemically, to find out if substances are given off to the cytoplasm and if so at what phases of cell life. I have made such a study of the nucleus of *Uroleptus Halseyi* a new species which I have described elsewhere and which is closely related to *Uroleptus mobilis* (CALKINS, 1929).

In both species of *Uroleptus* the new macronucleus arises after coujugation by a second mitotic division of the amphinucleus. In *U. mobilis* (CALKINS 1919) from four to six days are required for complete reorganization — in *U. Halseyi* only two to three days. The new macronucleus then divides twice, the new micronuclei, also, and their division is followed by division of the cell. Each

daughter cell now has two macronuclei which quickly divide to form eight. These eight constitute the usual, or possibly normal, condition and each is a distinct and independent nucleus with its individual membrane. Unlike the beaded nucleus of Stentor or Bursaria there is no continuous membrane with bead-like nodes.

In both species of *Uroleptus*, prior to division of the cell there is a fusion of all eight (or more) macronuclei and a condensation to form one, single, ellipsoidal nucleus which now behaves exactly as does the single nucleus of an ex-conjugant. Before this fusion and condensation however a considerable portion of each macronucleus is separated off and becomes distributed in the cytoplasm. This process for *U. mobilis* was described as follows (p. 299—303, Calkins 1919):

"The eight resting macronuclei of *Uroleptus mobilis* all have the same structure. They are densely granular, with a delicate membrane about them and at times, with a nuclear cleft (Kernspalt) characteristic of the hypotrichous ciliates. As to the significance of this nuclear cleft, I shall have something to say in a subsequent paper. It is not present in young cells after division, nor during conjugation, and disappears at an early stage of the division activities.

The two portions of each macronucleus, separated by the nuclear cleft, are apparently different in chromatin make-up, one portion being less dense than the other. At an early stage in division the chromatin granules of the less dense portion, concentrate into a single granule in each nucleus. Later, these granules are cast off, and are absorbed in the cytoplasm. This differentiation may be accomplished by further fragmentation of the remaining nuclear parts, resulting in from twelve to fourteen smaller masses. In all cases, however, after elimination of the chromatin granules, the nuclear fragments fuse to form a single elongate and irregularly wound nuclear mass. The granular contents condense, with shortening and loss of the irregularities, until a single, densely granular and massive, nucleus results. It is now ready to divide; it assumes an ellipsoidal form; its chromatin granules become arranged in lines running from end to end and it constricts in the center, forming a typical dumb-bell figure before there is any external sign of cytoplasmic division.

The daughter nuclei next divide to form four nuclei, and these in turn form eight, four of which belong to the anterior half, four to the posterior. The lines of densely staining chromatin granules are retained throughout all of these division stages, a characteristic

dumb-bell nucleus being formed at each stage. During the division from four into eight the cell constriction deepens in the division zone, and the cell divides, the two daughter cells having four nuclei each. The four nuclei finally divide once again, after division of the cell and after separation, and the eight nuclei, characteristic of the normal vegetative phase, are formed. At each nuclear division the connecting strands are severed so that the daughter nuclei are not connected by any linin or chromatin material."

In *Uroleptus Halseyi* the history of the macronucleus is essentially the same with minor differences however which will be discussed in the present paper in connection with the history of the nuclear granules. The number of macronuclei in this latter species, while frequently only eight, is more often ten to sixteen and in some cases the number runs up to twenty five or twenty six. There is however, the same type of elimination from each nucleus and the same type of nuclear fusion and condensation resulting in a single macronucleus at times of division.

Little exact knowledge of the chemistry of protozoan nuclei is on record. We have been accustomed to assume that substances in protozoan nuclei which stain in the same way as do fish spermatozoa or other chromatin-rich cells which can be obtained in large quantities, are ipse facto the same in chemical make up. In a general way this is probably true and we are perhaps justified in assuming that a macronucleus contains a large percentage of nucleoprotein. The nucleoproteins comprise a vast group of substances however, which invariably consist of some form of nucleic acid combined with some form of organic base such as histone or protamine. Such substances are characterized by their affinity for basic dyes a condition which has led to the unwarranted belief that all substances which stain with basic dyes are chromatin (see PRATJE 1920). Any acid substance in the cell should give a similar staining reaction and the effect of a basic dye is no proof of the presence of nucleic acid. The nucleic acids in turn are numerous, varying with the amount of phosphoric acid and the organic base with which they are combined. Thus nucleic acid from the thymus gland according to STEUDEL (1912), consists of a condensed phosphoric acid combined with four glucoside molecules each of which is made up of a carbohydrate (hexose) and one base either guanine, cytosine, thymine or adenine, the whole having an empirical formula of C_{43} H_{61} N_{15} P_4 O_{34} (PRATJE 1920). Other nucleic acids such as those from the pancreas or from muscles are essentially simpler consisting of one

molecule of phosphoric acid, a pentose and a purine base (e. g. guanylic acid).

In connection with the chemistry of protozoan nuclei Reichenow states: In general we have recourse only to microchemical methods whereby results are often unsafe and lead to contradictory conclusions. These methods consist essentially in comparing the solubility of certain structures of the cell in acids, salt solutions or alkalies and identifying them with known substances which are soluble in these reagents. Unna employs a process which he terms chromolysis consisting of the use of a given stain before and after testing the solubility with such reagents. If the structure or substance stains before but fails to stain after using the reagent he concludes that it is the same in chemical nature as a substance which is known to dissolve in the reagent used. With this method Unna and Thielemann found no nucleoprotein in the nuclei of Amoebae and Trypanosomes the main albumen portion according to them being protamine in Amoeba and histone in Trypanosoma (Doflein, Reichenow 5th Ed. p. 17). This conclusion however is contradicted by Oesterlin, while M. Robertson (1927) has demonstrated the presence of nucleic acid in Trypanosoma by the Feulgen nucleal reaction test.

A distinct advance in technical methods for the identification of chromatin is afforded by the so-called Feulgen nucleal reaction. This is based upon the macrochemical Schiff's test for true aldehydes and in its application upon the principle of acid hydrolysis of the nucleic acid molecule whereby the purine bodies are split off thus freeing the aldehydes. Basic fuchsine in a solution of sodium bisulphite and hydrochloric acid followed by a washing solution of sodium bisulphite and hydrochloric acid, gives a lavendar or violet color to the aldehyde components of the chromatin while nothing else is stained.

This method has been used on Protozoa with successful results by Feulgen, Bresslau, Reichenow, M. Robertson and others and has proved a valuable asset in our technique.

Methods Employed.

The Feulgen method should not be depended on for the whole story of the nuclear structures. Much may be learned from the use of the more delicate basic stains as well as by iron haematoxylin but not much is gained by the use of vital nuclear dyes

such as methyl green. The results given in the present paper are based upon the following technical methods.

A. Fixation. To give uniformity of treatment and for comparison with the earlier work on *Uroleptus mobilis* I have used saturated bichloride of mercury in 95 % alcohol. Unless otherwise stated this method has been used throughout.

A cover glass is smeared with egg albumen and specimens of Uroleptus, preferably from an agglomeration, are picked up in a capillary pipette (care being taken to get a minimum of water) and deposited in the center of the smeared surface. The drop of water containing the organisms should not be more than three sixteenths of an inch in diameter, less if possible. A drop of killing fluid is then dropped from a height of an inch onto the organisms. The liquids spread out in all directions thus stranding the organisms, which are caught in the albumen now coagulated by the fixing mixture. Crystals begin to form very quickly but before they cover the surface the cover glass must be immersed in a container filled with the killing fluid. They remain in the killing fluid for at least half an hour after which they must be left in 95 % alcohol for an hour or more. This is usually long enough to remove all traces of the mercuric bichloride, if not the cover glasses should be left for a few minutes in 70% iodized alcohol. After this they may be kept indefinitely in clean 70% alcohol from which they may be removed for any type of treatment.

B. Staining. The staining methods employed have been confined in the main to a) iron haematoxylin; b) the Borrel stain; and c) the Feulgen nucleal reaction. Other stains have been used but most of the preparations have been made with these.

a) Iron haematoxylin. I have found that the long haematoxylin method is unnecessary. Equally intense staining and clear differentiations in a fraction of the time are obtained by use of the following modifications: The cover glasses are placed from water in a jar of 4% iron alum which is heated to 50° C in a sterilizer. They remain here at this temperature for 5 minutes when they are transferred to a jar containing 1% aqueous haematoxylin also in the sterilizer at the same temperature. At the end of five minutes the organisms are jet black and require from three to five minutes for de-staining. This is done in a 2% solution of iron alum, used cold, and differentiation is controlled under the misroscope. Thus after fixation a preparation that is ready to study can be made in 15 minutes.

Uroleptus Halseyi is so filled with mitochondria that unless the latter are removed the nuclear structures cannot be made out with any accuracy. They may be easily removed however by half an hour immersion in a jar of 20% acetic acid in 95% alcohol after which the nuclear structures are clearly differentiated with iron haematoxylin.

b) The Borrel Stain. This combination of stains gives a beautiful differentiation of what we usually term the chromatin content of the cell. It is made up in two solutions 1. a saturated aqueous solution of magenta (Diamant fuchsin) and 2. a mixture of 3 parts of a saturated aqueous solution of indigo carmine and 2 parts of a saturated aqueous solution of picric acid. The blue and yellow thus mixed give a delicate but intense acid green stain. In using this combination the cover glasses are run down to water and left in the magenta solution for twenty minutes. They are then rinsed thoroughly in water and, for Uroleptus, are left for not more than two minutes in the green mixture. They are again rinsed in water until the green disappears and the cover glass appears red. The red comes out in alcohol so that the preparations must not be neglected at any stage although there is no need of precipitate action at the risk of improper dehydration. The least trace of acid however in the alcohols is fatal to a good preparation. In 100% alcohol the visible red must have disappeared entirely and with a return of the green. In such preparations the chromatin elements are stained a bright red while everything else stains green. The time of exposure to the red and to the green must be determined for each type of organism the great majority being overstained with the green but others may show a predilection for the red. For the former a prolonged washing in water may be the remedy, for the latter, a more leisurely journey through the alcohols.

c) The Feulgen nucleal reaction. The technique employed by different investigators varies somewhat as to details but the solutions used are the same. One of these
1 is the staining solution; # 2 the washing solution.
1 Solution: (Use after pink color has disappeared).

1.	Basic fuchsine (Magenta, Grübler)	0. lgr.
2.	Distilled water	100. cc.
	Use heat if necessary: when cool add	
3.	Sodium bisulphite 30% solution	3. cc.
4.	After 1 hour add pure HCL	1. cc.

2 Washing solution

1. Sodium bisulphite 10% solution	10. cc.
2. N/1 HCL	10. cc.
3. Tap or spring water	80. cc.

MURIEL ROBERTSON (1927) fixes *Bodo* and *Trypanosoma* species in SCHAUDINN'S fluid, hardens in 90% alcohol for 24 ± hours. Then from water she places the film in N/1 HCL for a moment and then immediately into N/1 HCL at 60° C for from 5 to 7 minutes. The preparation is then quickly rinsed in water and placed in the staining solution # 1. Here it is left for from $1^1/_2$ to 24 hours at room temperature, the time depending on the organism. After this period the preparation is washed thoroughly in 3 changes of solution # 2, and then in running tap water for half an hour. She counterstains, dehydrates, and mounts in the usual way.

REICHENOW (1927) fixes his material preferably in sublimate-alcohol and from water places it immediately in N/1 HCL at 60° for only 4 minutes. Then after rinsing in water it is placed for a period varying from 1 to 3 hours in solution # 1. For washing (solution # 2) he dilutes solution # 2 by making it up with 200 cc of water instead of 80 cc. In other respects his method is the same as ROBERTSON'S.

In applying the FEULGEN method to *Uroleptus* I find the best results come from the following procedure. Cover glass smears are prepared with sublimate alcohol as described above and run down to water. They are then placed in N/1 HCL at 60° C for 7 to 9 minutes, rinsed in water and left in the fuchsine solution # 1 for 24 hours at room temperature then washed thoroughly in three changes of the washing solution. After subsequent washing in fresh water they are run through the alcohols and mounted in the usual manner. An exposure of only eight hours in the fuchsine solution, the rest of the process as above, results in a feeble reaction as indicated by a pale lavendar color of the nuclei, but after 24 hours or even 48 hours in the fuchsine the nuclei show the reaction intensely and the color is much deeper and of a rose red tint.

Observations.

I will first describe the structure of the macronuclei of *Uroleptus Halseyi* as revealed by a typical vegetative stage. There are characteristic reactions after the different methods employed which can be followed more easily by first describing the structures as shown by the usual iron haematoxylin stain.

1. Iron haematoxylin. The nuclei are elongate and typically ellipsoidal in optical section but owing to their plasticity they conform to the shape of the cell and may assume various forms (Pl. 12 Fig. 9). They vary in size from 10 μ in spherical forms to 20 μ in the more elongate types and the size varies inversely as the number in the cell.

Each nucleus may have a delicate nuclear membrane. It does not stain, however, and cannot be made out as a double contoured structure, the only evidence of it in iron haematoxylin preparations is the orderly arrangement of the peripheral nuclear granules which in turn may be due to their being embedded in a more or less solid matrix.

Two types of nuclei, differing according to the make-up of the granular contents, are characteristic of these vegetative nuclei. In one type (Fig. 9) the granules are of uniform size, spherical and equally distributed in the nucleus. These are always chromatin and will be called the C-granules. They are fewer in number and more separated in the terminal nuclei both anteriorly and posteriorly while in the central nuclei they are so numerous and so densely packed that the nucleus appears homogeneous. In the second type of nucleus the granules are of diverse size and there are two types, C-granules and X-granules. The bulk of the nucleus is filled with uniform, spherical, C granules but here and there in their midst are from two to five larger and more conspicuous spherical granules which usually have a halo about them (Pl. 12 Fig. 10). These larger granules stain intensely black and will be referred to as the X-granules. These are widely known as the Binnenkörper (e. g. Reichenow).

2. The Borrel Stain. There is a very delicate equilibrium between the basic and acid constituents of this stain on the one hand and the acid and basic substances of the cell of Uroleptus on the other. This is clearly demonstrated by the reactions of the components of the nucleus during this vegetative stage. The macronuclei are easily stained green by the acid stain if more than a two-minute exposure is given. With short exposure they stain red with the magenta and the first type of the haematoxylin series above is common. Here the uniformly distributed granules are stained a brilliant magenta red (Pl. 11 Fig. 1). Again the terminal nuclei show fewer granules than do the central nuclei which appear uniformly red so closely are the granules packed together. With the X-granules however, the case is different. These large, internal, granules usually stain green when the surrounding granules are

red (Pl. 11 Fig. 3), but, like the C-granules they may also stain red (Pl. 11 Fig. 2). If however, the nuclear granules stain green by reason of a longer exposure to the acid component of the Borrel stain then these X-granules may stain red (Fig. 4).

Postponing for the moment a consideration of the significance of this discrepancy the facts indicate that these X-granules are not composed of the same chemical substance as are the C-granules. The possibility of their being volutin grains comparable with the cytoplasmic granules of bacteria, flagellates and Sporozoa (see REICHENOW, VAN HERWERDEN, DOBELL and GUILLIERMOND), was considered. This seemed reasonable in view of the fact of the wax and wane of volution in the presence and absence of phosphorus in the medium. On trying out MEYER'S test for volutin, however (Methylene blue followed by 1% sulphuric acid), there was not a grain of volutin in the macronuclei nor alsewhere in the cell although flagellates and bacteria responded perfectly. It appears therefore that these X-granules while possibly chromatin, are not the same in chemical composition as the other chromatin granules and that they are approximately neutral in nature. Why do they stain green however when the chromatin stains red with BORREL and vice versa? They disappear after prolonged treatment with 20% acetic acid alcohol which would indicate a lipoid characteristic. This also shows that they are not chromatin, a conclusion borne out by the FEULGEN reaction.

3. The FEULGEN reaction. This method is generally admitted to be the best test for chromatin that we have and nothing else in the cell gives the reaction. It is not dependent upon destaining but, if the solutions are properly made, the reaction is reliable.

With *Uroleptus Halseyi* not only the micronuclei but all of the macronuclei as well give a positive reaction. In the resting condition of the vegetative stages the micronucleus appears as a homogeneous solid the entire micronucleus giving the reaction. The macronuclei on the other hand are either uniformly filled with granules of the C type which give the typical FEULGEN reaction, or from two to many vacuoles may be present amongst the C granules (Pl. 11 Fig. 7). The fate of the X-granules is interesting. They are evidently hydrolyzed and their former sites in the nucleus are now indicated by the vacuoles in each macronucleus. This result coincides with some of the stages in which the X-granules stain differently from the C-granules with the BORREL mixture and indicates a combination of acid with some different substance than that which shows the FEULGEN reaction.

In the resting macronucleus therefore, we have at least two kinds of granules which ordinarily take the basic dyes. The largest of these, X-granules, are internal and disappear completely after the Feulgen treatment. The bulk of the nuclear material, C-granules, are always demonstrable by the Borrel mixture, the Feulgen reaction or by the iron haematoxylin stain. The fate of these granules will be followed in the next section.

B. The Macronuclei in Division. Division of the macronucleus of Uroleptus cannot be briefly summarized as a "process of simple division". The fact that a mitotic figure and clearly-defined chromosomes are wanting has led to neglect of this important cell-organ. Far from being negligible it is on the contrary probably the most important element of the cell in matters of metabolism, reorganization, and continued cell life. Its division is preceded by far-reaching changes both in the nuclear make-up and in the composition of the cytoplasm and these changes are clearly indicated by use of the stains employed in this investigation.

1. Iron haematoxylin. The appearance of the X-granules is really the first sign of preparation of the macronuclei to divide. These granules collect in the vicinity of the center of the nucleus and a cleft appears in the zone about them (cf. Pl. 11 Fig. 5 a and b). This cleft is the well-known Kernspalt or nuclear fissure characteristic of hypotrich macronuclei. The X-granules may be combined in one large granule directly in this fissure or there may be two or more, or some residual granules may be in with the chromatin granules of the nucleus in both parts separated by the cleft or in only one of them (Pl. 11 Fig. 5). The smaller portion of the nucleus has very fine, uniform chromatin granules which I will call the D-granules and is always cast off together with the X-granules in the cleft. The remainder of the nucleus with uniform C-granules is now ready to fuse with adjacent nuclei which have had a similar history. After the haematoxylin stain these stages are not so spectacular as they are after Feulgen or the Borrel stain, but they are clear cut and convincing. The fate of the discarded portions cannot be followed for they quickly break up and are lost amongst the gray-staining granules of the cytoplasm.

Fusion of the nuclei results first in an elongate skein-like complex with many protuberances and irregularities (Pl. 11 Fig. 6). This shortens and condenses until a perfectly homogeneous densely granular division nucleus results. In this the chromatin granules may be arranged linearly but this arrangement is not evident until

the nucleus begins to divide when the linear arrangement can be seen (Pl. 12 Fig. 11). The single nucleus usually divides twice prior to division of the cell when each daughter cell receives two; these two in turn divide twice to restore the normal nuclear relations.

2. The BORREL Stain. The X-granules become much larger prior to the formation of the nuclear fissure and stain bright green (Pl. 11 Fig. 5). One or sometimes two large ones take up a position at about one-third of the length of the nucleus from one end (Pl. 11 Fig. 5 a). At this zone the nuclear fissure appears as a sharp cleavage across the entire nucleus (Pl. 11 Fig. 5 b). The red-staining C-granules are clearly separated into two sets and the larger X-granules to which others are now added, become closely applied to the cleavage surface of the smaller nuclear fragment (Pl. 11 Fig. 5 c). Other X-granules which may be present in either fragment are now actually cast out from the chromatin aggregates and are soon lost in the cytoplasm (Pl. 11 Fig. 5 d).

The smaller nuclear fragments are usually oriented in the same direction in all of the nuclei and all are cast off with the attached substance of the X-granules into the cytoplasm where they soon disintegrate and disappear. Their chromatin granules are much finer than the C-granules and soon lose their affinity for the magenta stain after they are cast off and added to the number of cytoplasmic granules. These are referred to as the D-granules.

Shortly after this "purification" has taken place the nuclei unite. This process begins first with the terminal nuclei and fusion progresses towards the central nuclei (Pl. 11 Fig. 6). The phenomenon is not unlike the union of two rhizopods in plastogamy but the chromatin granules, now berett of all X-granules, assume a linear arrangement (Pl. 11 Fig. 6). With the shortening of the chain of fused nuclei the chromatin granules probably fuse although this could not be determined by observation but the mature nucleus consists of closely-packed, deeply-staining rods of chromatin the actual division of which could not be observed either in total mounts or in sections. These may be referred to as the macronuclear chromosomes.

This dense nuclear structure persists until the four macronuclear divisions are completed to re-establish the 8 macronuclei in each daughter cell. The X-granules appear only with re-establishment of metabolic activity.

3. The FEULGEN reaction. Since the X-granules do not give the characteristic reaction of chromatin with this method it is of little help in connection with the preparation for nuclear fusion.

It is significant however that not only are vacuoles present in the nucleus (Pl. 11 Fig. 7) but all evidence of the part played by the X-granules is obliterated (Pl. 11 Fig. 8). In one or two cases however I have noted the presence of an indefinite refractile spherule in the clearly-defined nuclear fissure. After the smaller fragment of a nucleus is cast off there is no evidence of it in the cytoplasm, in other words the nucleic acid character is lost. Evidence of this loss is furnished also by the slight reaction or none at all on the part of this fragment before it is cast off and while the nuclear fissure is present (Pl. 11 Fig. 8).

C. The Macronuclei during Conjugation. It is well known that profound changes in the cell occur during conjugation and that these changes are continued in the exconjugant until reorganization is complete. Little is known about the cytoplasm during this period of break-down and reconstruction but the general history of the nuclei is established. The micronucleus undergoes two divisions shortly after fusion of the two cells. These are usually regarded as meiotic divisions the second of which is usually a reducing division whereby the number of chromosomes is reduced to one half. A third division then follows resulting in two pronuclei one of which migrates into the other individual the other nucleus remaining stationary. These phenomena are mutual hence there is an interchange of pronuclei, the migrating nucleus uniting with the stationery nucleus in each case. The fusion nucleus, or fertilization nucleus, or amphinucleus as it is variously called now divides once or twice before the conjugating animals separate. In some ciliates one of the products of the first division becomes a micronucleus, the other a macronucleus. In other cases one of the products of the second division forms the new macronucleus while another forms the micronucleus and two degenerate. In still other cases the amphinucleus divides three times, four of the final products forming macronuclei, four micronuclei. Whatever the detailed method, the fact appears to be universal that the new macronucleus does not come from a micronucleus as usually stated but from the amphinucleus.

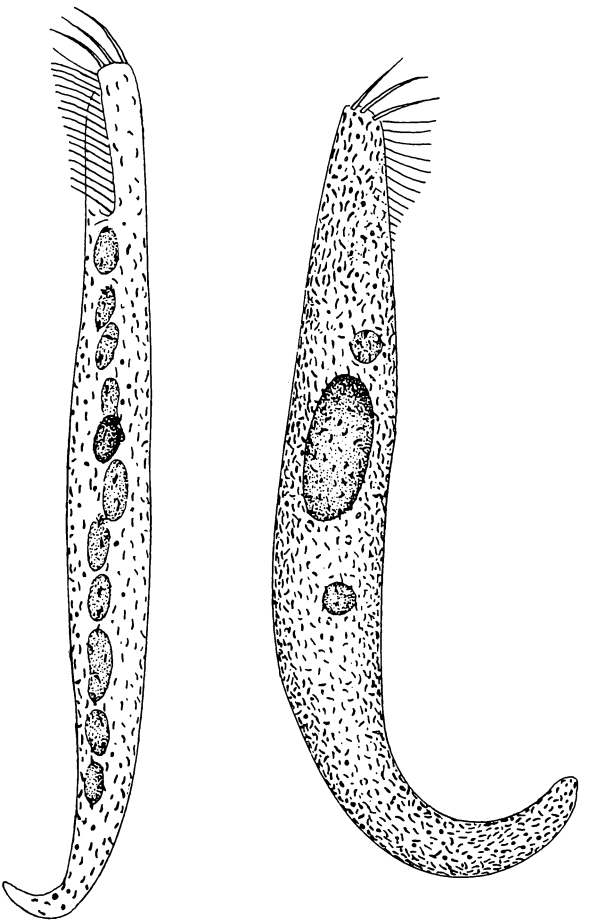
The macronucleus of each of the original individuals meanwhile undergoes degeneration and complete disappearance during this period of reorganization and our problem here is to describe the processes of degeneration and of transformation of the amphinuclear fragment into a new macronucleus.

1. Degeneration of the Macronuclei. At the outset of conjugation the structure of the macronucleus is practically the

same as in the usual vegetative condition. The pictures are the same whether iron haematoxylin, BORREL, or FEULGEN is used. With none of them is there any evidence of the X-granules and there are no vacuoles with the FEULGEN. This condition continues with minor variations until the parachute phase of the micronucleus when large granules appear in the macronuclei. These stain black with iron haemotoxylin (Pl. 2 Fig. 12) or red with BORREL and give the characterlstic vacuoles after FEULGEN (Pl. 12 Fig. 13). This condition persists throughout the first division of the micronucleus but the intensity of the staining reaction decreases (Pl. 12 Fig. 14). At the period of the second meiotic division of the micronucleus, the small chromatin granules are no longer evident. In their place we find a smaller number of much larger granules varying in size from two to four times the volume of the earlier small granules. These larger granules tend to collect in groups leaving relatively large spaces in the nuclei and giving the latter the effect of a vesicular structure (Pl. 12 Fig. 15). At this stage and throughout the period of interchange the general staining reaction (Pl. 12 Fig. 16) is feeble and with the BORREL mixture the macronuclei are green. A very characteristic effect at this period is the change in the arrangement of the macronuclei. They are no longer strung out in a row of nuclei as in vegetative and division stages but are clumped together in irregular groups from the center to the posterior end (Pl. 12 Fig 17). There is no fusion however, each nucleus remaining distinct and definite. In some cases there is a tendency to fragment and small chromatin masses may then be seen in the cytoplasm.

After the individuals have separated and the new nuclei are in the process of organization, the last vestiges of the old macronuclei disappear. These final stages are not demonstrable by the BORREL stain nor by the FEULGEN method but come out clearly with iron haematoxylin. We have described above the vesicular character of the old nuclei during the later stages of conjugation and with a tendency to fragment (Pl. 12 Fig. 17). In the exconjugant stage this fragmentation continues until the old nuclei have disappeared as such leaving a great number of minute nuclear elements some of which may still be picked out by the FEULGEN method. These fragments ultimately give rise to a still greater number of minute spherules which are most abundant in the posterior half of the cell (Pl. 12 Fig. 21). At one stage these spherules stain lightly with iron haematoxylin but not uniformly, and they ultimately lose even this slight tinge. In these later stages each spherule is surrounded by

an intensely stained cortex which breaks up into a number of saucer-shape, or in optical section crescentic, black bodies (Pl. 12 Fig. 21). In this manner the posterior ends of the ex-conjugants become filled with large deeply-staining granules which are distributed throughout the cell at a later stage (Text-Fig. 1).



Textfig. 1. The so-called mitochondria of *Uroleptus Halseyi* Calk. At the left, a normal but small vegetative individual with ten macronuclei and one micronucleus The mitochondria are distributed throughout and are relatively scarce. At the right, an ex-conjugant almost ready for its first division. The macronucleus is single and filled with densely staining chromatin. There are two micronuclei. The mitochondria are distributed throughout and are abundant particularly in the posterior end. 700 : 1.

These black-staining granules appear to be identical with what I have described as the chondriosomes or mitochondria of the normal vegetative Uroleptus (CALKINS 1929). They are destroyed by strong acetic acid and become relatively scarce during conjugation, but the ex-conjugants contain them in enormous numbers, and they originate from the fragmented nuclei of the ex-conjugants. I am aware of the far-reaching significance of this observation if the bodies in question are mitochondria but I would reserve final judgment on the latter point until I have had an opportunity for further study.

2. Development of the new macronucleus. In a large number of ciliates the developing new macronucleus is readily seen in the living animal as a clearly defined spherical vesicle near the center of the body. This was known as the "placenta" in earlier publications. When fully developed it becomes invisible during life. During the early development it is impossible to stain it successfully with nuclear dyes but when fully formed it colors intensely with practically any basic stain. With FEULGEN it gives, at first, a characteristically faint reaction which however, increases in intensity as complete organization is attained. This indicates a gradual accumulation of nucleic acid which is scarce at first in the nucleus but is abundantly developed in later stages. During these stages of development minute granules in the otherwise homogeneous matrix of the nucleus are clearly stained by appropriate differentiation with the BORREL mixture and the iron haematoxylin stain (Pl. 12 Figs. 18 — 20). These granules become more numerous and more distinct with further development until they replace the former matrix of the nucleus and are now large, intensely staining and tightly packed granules characteristic of the macronucleus when ready for division (Pl. 12 Fig. 11). The processes of the first macronuclear division do not differ from those of the vegetative stages.

General.

The conception of chromatin has undergone many changes in the last fifty years. As originally used by FLEMMING (1880) the term was employed to specify the substance of the nucleus which appears to have a selective affinity for certain dyes, particularly carmine and haematoxylin. This was the colorable part of the cell and the word "chromatin" was a happy expression of this property. But "color" is as varied as the spectrum and other dyes, notably eosin, fuchsine etc. were known to color other substances of the cell quite as selectively as does carmine or haematoxylin, and such substances,

etymologically and with equal felicity, might have been called "chromatin". Under Ehrlich's influence these dyes were grouped into two sets, the "basic" and the "acidic" stains the former showing a selective affinity for substances of an acid nature, the latter for those of a basic nature. The term "chromatin" however, has persisted to the present day to connote the nuclear substances. But differences in the staining capacity of chromatin led to further attemps to specify different nuclear constituents and Heidenhain's terms "basichromatin" and "oxychromatin" were the result.

With the development of theoretical (Weismann et al) and experimental (Morgan et al) genetics, interest became focussed in chromatin as it appears in the formed chromosomes and in the chromomeres (with contained "genes") which enter into chromosome formation and are derived from chromosomes in the late telophase stages. If we rely solely on the staining test the basichromatin would seem to be absent from many types of resting cells or else the chemical make-up of chromatin changes with the relative wax and wane of the nucleic acid content. Boveri (1904) long since led the way to this conclusion: „Unter chromatischer Substanz verstehe ich also hier die Substanz, die uns in den Chromosomen vorliegt, und das, was im ruhenden Kern aus ihr wird oder was aus dem ruhenden Kern sich wieder zu den neuen Chromosomen zusammenzieht. Ob sich diese Substanz der Chromosomen selbst wieder als irgendwie zusammengesetzt erweist, dies bleibt hier gänzlich unberücksichtigt. Es mag also sehr wohl sein, das hier unter ‚chromatischer' Substanz auch Teile mit inbegriffen werden, die im ruhenden Kern gerade als ‚chromatische', als ‚Linin', Plastin oder anderswie bezeichnet werden; ja es wäre für unsere Betrachtungen ganz gleichgültig, wenn das, was durch den ruhenden Kern hindurch die Kontinuität der Chromosomen vermittelt, überhaupt gar nicht ihr färbbarer Bestandteil wäre" (Boveri, 1904, p. 2. Quoted from Bělař, 1926, p. 241). The chemical aspects of this point of view are more recently expressed by Wilson as follows: "All this falls in line with the assumption that during the vegetative activities of the cell the protein bases of the nucleus increase in quantity, the acidic character of the chromatin (and hence its basophily) correspondingly diminishing as the affinities of the nucleic acid radical are more completely satisfied. Whether this means an actual increase in the protein components, or a diminution of the nucleic acid, can only be surmised" (The Cell, 3rd Edition, 1925, p. 652). Again: "So far as the staining reactions show, therefore, it is not the basophilic component (nucleic

acid) that persists, but the so-called "achromatic" or oxyphilic substance. The nucleic acid component comes and goes in different phases of cell activity, and it is the oxyphilic component that seems to form the essential structural basis of the nuclear organization" (ibid. p. 653).

Still more recently BĚLAŘ designates chromatin as follows: „Bei der Unmöglichkeit, das Chromatin mikrochemisch exakt zu charakterisieren, müssen wir uns begnügen, mit diesem Namen alle diejenigen Strukturen des Ruhekerns zu belegen, die sich mit basischen Teerfarbstoffen intensiv färben, soweit die morphogenetische Analyse der Kernteilung nicht zeigt, daß sie am Aufbau der Chromosomen unbeteiligt bleiben. Eben diese morphogenetische Analyse zwingt uns aber andererseits, in vielen Fällen Strukturen, die sich färberisch entgegengesetzt oder neutral verhalten, ebenfalls als Chromatin zu bezeichnen, sobald nämlich der Nachweis erbracht ist, daß sie genetisch mit den Chromosomen zusammenhängen" (BĚLAŘ, 1926, p. 241).

From the above citations it appears, that chromatin today is still more of a conception than a definite substance. The FEULGEN reaction however has now given us a new method for studying the microchemistry of chromatin and when perfected, its use should throw considerable light on the different aspects of chromatin change. Some progress has already been made in the study of protozoan nuclei by this method (FEULGEN, BRESSLAU, M. ROBERTSOHN et al) and in the present paper I have endeavoured to apply it in the study of different stages in the history of the chromatin of *Uroleptus*. I am at a loss however, to interpret some of the reactions that have been observed.

The underlying principle of the reaction is the acid hydrolysis of the nucleic acid molecule whereby the carbohydrate component is freed from the combination with phosphoric acid and organic bases (guanin, adenin etc.) all of which together make up the molecule of thymonucleic acid (STEUDEL, 1912). Such nucleic acid in turn is one of the components of nucleo-protein, the other being an albumen base (histone, protamine). After hydrolysis the carbohydrate component is stained violet or lavendar by basic fuchsine (magenta or diamant fuchsine) in the presence of an acid (HCL) and sulphur (sodium bisulphite) which is the typical reaction of the macrochemical SCHIFF'S test for true aldehydes.

In analyzing the results obstained by use of the FEULGEN reaction as well as from other methods of staining, I am compelled

to start with the acknowledgment of my complete ignorance of the chemical nature of the substances in the macronucleus of *Uroleptus Halseyi* which form the matrix in which the nucleic acid substances develop. Beginning with the products of the second division of the zygote nucleus after conjugation we find four nuclei all of which stain moderately with the usual nuclear dyes and all of which give a positive but faint Feulgen reaction. As reorganization progresses two of these four nuclei condense and stain more intensely with all methods employed. These become the two micronuclei of the exconjugant and will not be considered further in the present paper. One of the remaining two nuclei degenerates and disappears in the cytoplasm (in one case however, I found that it also formed a "placenta"). The fourth nucleus forms the new macronucleus of the ex-conjugant (Pl. 12 Fig. 18). Instead of increasing in staining intensity as do the new micronuclei, it becomes pale and homogeneous in structure. Furthermore instead of decreasing in size it swells until it is a huge spheroidal structure and the most conspicuous object in the organism. It is now the so-called "placenta" of the re-organizing ex-conjugant. The substance of this nucleus is homogeneous at first but even at an early stage exquisitely minute points of staining material are seen to be scattered through it. These are the beginnings of the chromatin granules of the new nucleus. It is difficult to illustrate this stage in any way that will give a satisfactory picture of the phenomenon. With the Borrel stain the minute granules appear as shining red dots in the drab and homogeneous matrix of the nucleus; with iron haematoxylin they are black, and after the Feulgen reaction they are equally conspicuous as violet points of substance in the unstained matrix. These granules are not formed on the periphery nor in segregated groups but are dispersed throughout (Pl. 12 Fig. 18).

With increase in size of the nucleus the granules become larger and more conspicuous (Pl. 12 Fig. 19, 20) until ultimately what was the matrix gives place to a multitude of rod-like chromatin granules forming the characteristic "massive" type of macronucleus ready for its first division (Pl. 12 Fig. 11).

What is the substance of this matrix? The Feulgen reaction shows that it is not the aldehyde component of nucleic acid. Possibly it is an organic base (histone or protamine) of the nucleo-protein. Whatever it is we find the nucleic acid forming in it and ultimately replacing it. The entire phenomenon appears to be, not evidence of a low percentage of nucleic acid, but evidence of its actual manufacture.

The chromatin of the macronucleus, once formed, persists throughout the vegetative stages, growing and dividing with each cell division but the nucleus never reverts to the condition of the original homogeneous matrix. Ultimately, with endomixis or with conjugation, the macronuclei degenerate, the chromatin loses its staining capacity and shows only a pale FEULGEN reaction, and finally disappears in the cytoplasm.

During this vegetative history however, differentiations appear with every cell division. Bodies (X-granules) appear amongst the chromatin granules. With iron haematoxylin these stain like the chromatin granules and would seem to be chromatin (Pl. 12 Fig. 10). But they also stain with the acid component of the BORREL stain (Pl. 11 Fig. 3) and they disappear entirely with the FEULGEN treatment (Pl. 11 Fig. 7), hence they cannot be chromatin. What they are is problematical but they form part of the substance of the nucleus that is consigned to the cytoplasm at the time of division. With them about one-third of the entire chromatin content of the macronuclei is likewise shed into the cytoplasm, always with a loss of the constituent nucleic acid. The single division nucleus which results from fusion of the residual nuclei is loaded with chromatin, compact and densely-staining, which results from concentration and not from origin de novo as in the ex-conjugant.

Finally, what appears to be the last scene in the drama of the macronucleus is the aggregate of groups of vesicular structures after the nuclei have disappeared as such. The cortex of each vesicle stains black with iron haematoxylin and this cortex fragments into a number of crescentic or rod-like bodies which completely fill the cell of the ex-conjugant (Text-Fig. 1 and Pl. 12 Fig. 21). These bodies disappear after treatment with acetic acid, after the FEULGEN treatment, and after exposure to ultra-violet rays (CALKINS 1929). They appear to be of a lipoid nature and, if they are mitochondria as they seem to be, they are probably phospho-lipins in which the source of phosphorus might well be some transformation of the phosphoric acid of the erstwhile chromatin.

Summary.

1. There are from eight to twenty six independent macronuclei and one occasionally two, micronuclei, in *Uroleptus Halseyi* CALK.
2. At division a portion of each macronucleus is discarded and probably plays some role in the activities of the cytoplasm.

The remainders fuse to form one large, homogeneous, division nucleus.

3. During conjugation the macronuclei remain independent, lose their staining capacity, and disappear in the cytoplasm.
4. A new macronucleus is formed from one of the products of the second division of the zygote nucleus in the ex-conjugant.
5. If chromatin is defined simply as a nucleic acid holding substance giving rise to chromosomes, then there is no chromatin in the new macronucleus during its early stages. This is demonstrated by its failure to stain with basic dyes and by its failure to give a positive result in the Feulgen nucleal reaction.
6. The new macronucleus swells enormously forming the so-called placenta which is visible during life. It is filled with an unstainable substance which forms the matrix in which chromatin granules are manufactured.
7. The chromatin granules first appear as minute points distributed throughout the matrix. They stain black with iron haematoxylin, red with the Borrel mixture and violet after Feulgen.
8. With further development of the macronucleus the chromatin granules increase in size and number, and because of them, the nucleus as a whole begins to stain, faintly at first, intensely later.
9. Ultimately the nucleus is filled with intensely staining, large chromatin granules which now have replaced the substance of the matrix. The nucleus is no longer visible during life.
10. The macronucleus divides twice before the cell divides. Each daughter cell receives two macronuclei, and these divide twice again to form the typical number of independent macronuclei of the vegetative individual. Other divisions are erratic giving rise to a variable number of macronuclei of the adult.
11. The chromatin of each nucleus is in the form of small, uniform granules which are equally distributed throughout. These are designated C-granules.
12. With the approach of a division period a new type of granule appears, the so-called Binnenkörper of Reichenow. These stain black with iron haematoxylin, green with the Borrel mixture, and are entirely dissolved out by the Feulgen reaction. These are designated X-granules.

13. One large X-granule marks the zone in which the nuclear cleft (Kernspalt) will appear. There may be other smaller X-granules in a nucleus, if so they are discarded into the cytoplasm.
14. The nuclear cleft appears to be formed through the activity of the X-granule the substance of which forms a plate on the surface of the fragment to be discarded.
15. The chromatin of this fragment consists of granules-D granules-which are much finer than the C-granules. The D-granules, with the X-granules are discarded into the cytoplasm where they soon lose their identity.
16. The residual nuclei now contain only C-granules. After fusion of these residual nuclei each C-granule forms a rod-shape, intensely staining element which may be called a macronuclear chromosome.
17. During conjugation X-granules are formed at the outset but soon disappear. The C-granules enlarge thus indicating the first stage of degeneration. They gradually lose their staining capacity and ultimately disappear entirely. In some cases from two to eight deeply-staining spheres are present; these gradually fade away in the cytoplasm.
18. In the ex-conjugant the nuclear remains give rise to aggregates of vesicles, each vesicle with a deeply-staining (with iron haematoxylin) cortex which does not stain with the BORREL mixture and which disappears entirely with the FEULGEN reaction.
19. This cortex breaks up to form the crescentic or rod-form mitochondria which are very abundant in the ex-conjugant and which disappear with the FEULGEN treatment, with acetic acid, and with exposure to ultra violet rays.

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Explanation of Plates.

Plate 11—12.

Plate 11.

All figures represent a magnification of approximately 1000 diameters.

Fig. 1. Borrel stain. Three macronuclei from a vegetative form. C-granules present but no X granules.

Fig. 2. Borrel stain without the green component. Three macronuclei from a vegetative individual at an early stage in preparation for division. Red C-granules and red X-granules.

Fig. 3. Borrel stain. Three macronuclei from a vegetative individual at an early stage in preparation for division. Red C-granules and green X-granules. This is the usual reaction.

Fig. 4. Borrel stain. Three macronuclei in the same stage as in Figs. 2 and 3. The C-granules here are green as a result of long staining with the acid component, but the X-granules are red!

Fig. 5. Borrel stain. A complete set of macronuclei from an individual preparing for division. The C-granules are red, the X-granules green. The single micronucleus is a homogenous red. a, a nucleus with one large X-granule which lies in the region where the nuclear cleft will form. b, b, b Three nuclei with X-granules in the nuclear clefts. c, c nuclei in which the X-granules are spreading

out over the cleavage surface of the discarded segment; other X-granules are present but will be discarded (b, b). d, d Residual X-granules in the process of emission.

Fig. 6. Borrel stain. A characteristic stage in the fusion of the residual macronuclei; C-granules only are present.

Fig. 7. Feulgen nucleal reaction. Three macronuclei in the same stage as shown in figures 3 and 4. Minute vesicles show where the X-granules had been.

Fig. 8. Feulgen reaction. Five macronuclei in the same stages as those shown in Fig. 5. The nuclear clefts are present but X-granules are absent.

Plate 12.

Iron haematoxylin used throughout except in cases represented by figures 13, 14, 15 and 16. Except in Fig. 21 the magnification is approximately 1000.

Fig. 9. Three nuclei from a normal vegetative individual in which division activity had not begun. Uniform C-granules; no X-granules.

Fig. 10. Three nuclei from an individual in which division activity had begun. X-granules are present with the C-granules. (Cf. Fig. 3.)

Fig. 11. The single macronucleus with condensed chromatin ready for division.

Fig. 12. Early conjugation stage. The macronuclei have conspicuous X-granules and finely granular C-granules.

Fig. 13. Feulgen reaction. Same stage as that shown in Fig. 12, with spaces indicating the former presence of X-granules.

Fig. 14. Feulgen reaction. Three macronuclei at a later stage of conjugation.

Fig. 15, 16. Feulgen reaction. Groups of four and two macronuclei in clumps at the time of nuclear interchange and the first zygotic division (15). The C granules are much larger and less numerous than in the vegetative stages.

Fig. 17. Characteristic grouping of the macronuclei after interchange of pronuclei.

Fig. 18. A group of four nuclei, products of the second zygotic division of the amphinucleus. Two of these will become micronuclei; one (at right) will degenerate, and the fourth is the new macronucleus which is already swollen. Fine chromatin granules are distributed throughout its substance.

Figs. 19 and 20. Two later stages in growth of the new macronucleus.

Fig. 21. Portion of the posterior end of an ex-conjugant. The remains of two old macronuclei are shown as aggregates of vesicles. Various stages in the development of the mitochondria from the cortex of the vesicle are shown, also one new micronucleus. 1500 : 1.


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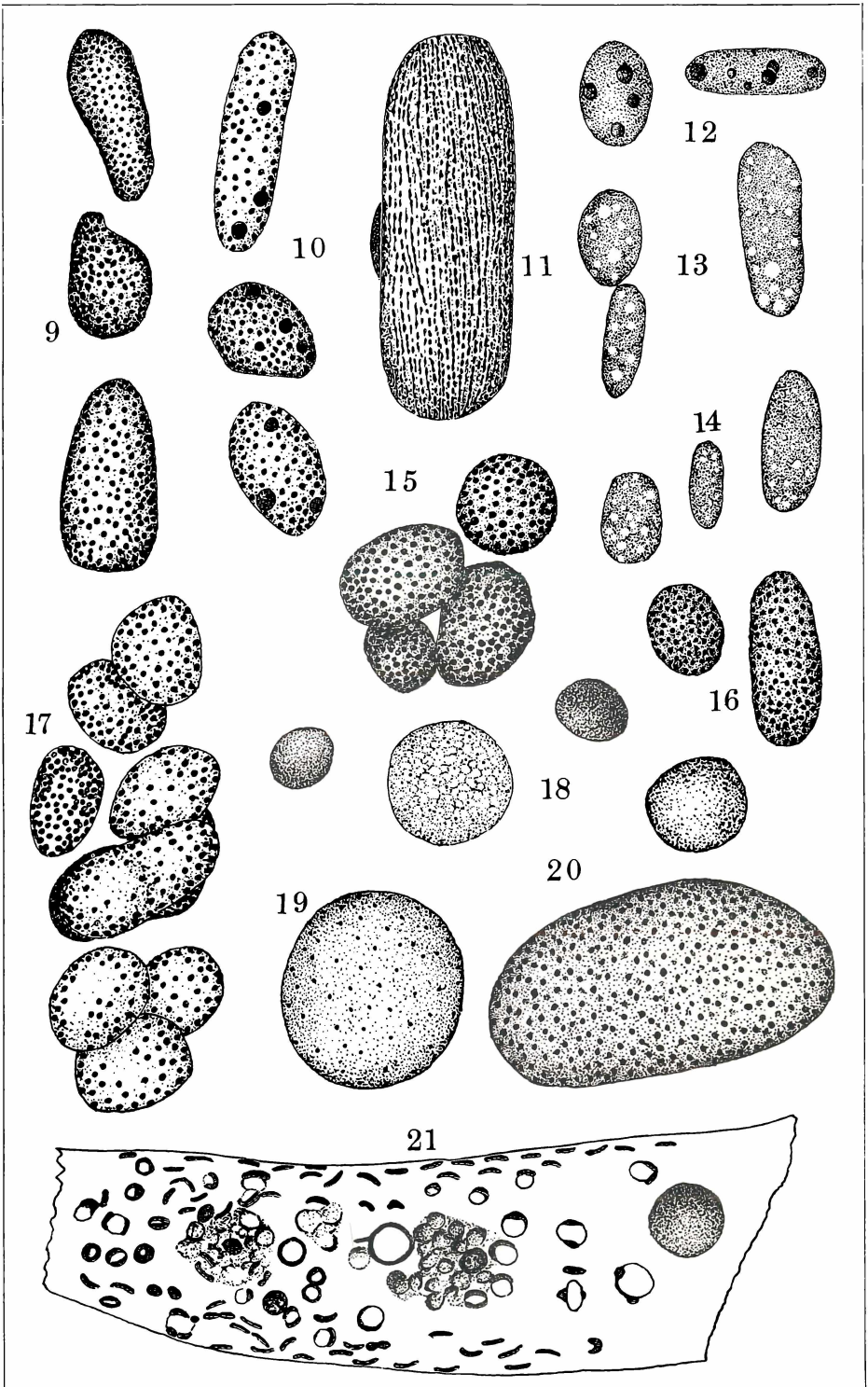

Calkins.

VERLAG VON GUSTAV FISCHER IN JENA.