

CYTOLOGICAL AND CYTOCHEMICAL
INVESTIGATIONS ON THE DEVELOPMENT
OF
SABELLARIA ALVEOLATA L.

BY

CHR. P. RAVEN, JANNY M. VAN BRINK, J. C. VAN DE KAMER
J. A. MILTENBURG AND F. H. SOBELS.

(STATION BIOLOGIQUE DE ROSCOFF, FRANCE, AND ZOOLOGICAL
LABORATORY, UNIVERSITY OF UTRECHT, NETHERLANDS)

VERHANDELINGEN DER KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN, AFD. NATUURKUNDE

TWEEDE SECTIE, DEEL XLVII, No. 1

1950

NORTH-HOLLAND PUBLISHING COMPANIE
(N.V. Noord-Hollandsche Uitgevers Mij.)
AMSTERDAM

Kon. Ned. Ak. Wet., Verh. Afd. Nat. (Tweede Sectie), Dl. XLVII, No. 1, p. 1—48, 1950

In recent years, many investigations have been made in which the displacements of cytoplasmic substances during the early development of eggs have been studied by means of cytochemical methods, in an attempt to obtain a better understanding of the processes bringing about ooplasmic segregation. This kind of researches has especially been initiated by the observations of SPEK (1930—34), though investigations of a similar nature had been made more incidentally before.

SPEK (1930, 1934b) studied the composition of *Nereis* and *Chaetopterus* eggs by means of vital stains having the properties of pH-indicators. Beginning some time after maturation or first cleavage, the animal half of the eggs stained with a tint corresponding to an alkaline reaction of the cytoplasm, whereas the vegetative half took on a colour as if its reaction were acid. At later development, the cells containing the "acid" cytoplasm give rise to the endoderm, whereas the "alkaline" blastomeres form the ectoderm. This "bipolar differentiation" of the cytoplasm was found back in the eggs of Teleosts (1933) and Cephalopods (1934a). In this case, the results of vital staining experiments were controlled by the injection of indicators into the eggs.

SPEK explains these observations in the following way: The cytoplasm of the egg is at first a mixture of positive and negative colloids; in the fertilized egg the particles of opposite charge are separated and accumulate at each pole of the egg. This is due to differences in electric charge of the egg surface in consequence of local differences in permeability to the ions of the external medium; in this way an electric field arises, in which the colloid particles are shifted to opposite poles. SPEK speaks, therefore, of a "kataphoresis in the living cell".

Though the observations of SPEK have been confirmed repeatedly also in other eggs, his interpretation of these results has met with many objections. First, serious doubts have been raised by many authors on the possibility to draw any conclusion on the pH of cytoplasm from the results of vital staining experiments. In the second place, his interpretation of the phenomenon of "bipolar differentiation" as a kataphoresis of cytoplasmic colloids has been questioned.

RIES and GERSCH (1936) studied the eggs of *Aplysia*. A "bipolar differentiation" could not only be demonstrated by the use of pH-indicators, but also with rH-indicators. However, according to the authors the phenomenon would not be due to a separation of colloid particles, but rather to an accumulation of the proteid yolk in the vegetative half of the egg. The "bipolar differentiation" is not specific for so-called "mosaic eggs"; it occurs also in eggs of the regulation type, whereas, on the contrary, several "mosaic" eggs do not show the phenomenon (GERSCH and RIES 1937; GERSCH 1939).

By combined centrifugation and vital staining experiments RAVEN (1938) showed that the interpretation of RIES and GERSCH is correct. The proteid yolk of the eggs of *Nereis*, *Chaetopterus* and *Aplysia*, when sedimented on one side of the egg by centrifuging, stains with an "acid" colour. "Alkaline" substances accumulate at the centripetal pole or in the hyaloplasm. The axis of stratification may make any angle with the primary egg axis; this does in no way influence the results of vital staining. A "bipolar differentiation" of the hyaloplasm was never observed. Hence, it was concluded from the experiments that the "bipolar differentiation" observed during normal development is due to the displacement of the inclusions of the cytoplasm. In the eggs of *Aplysia*, the substances displaced by centrifugal force did not remain in their new positions, but were redistributed throughout the eggs and assumed a normal position with respect to the egg axis in a rather short time. In those cases, however, where the distribution of the egg substances among the cleavage cells remained abnormal, a defective development ensued. Therefore, RAVEN expressed some doubts on the correctness of the conclusion, drawn by previous authors from centrifuge experiments, that the visible inclusions of the egg play no part in the determination of the cells. His results were confirmed by later experiments of RIES (1938) and PELTRERA (1941) on *Aplysia*. However, the latter author observed that an aberrant distribution of the egg substances among the blastomeres did not lead to the production of localized defects in the larvae, but rather to discordances in the relative sizes of organs. The egg substances do not represent definite "organ-forming substances", but the development of the parts of the embryo is governed by "cytochemical equilibria" in which various substances play a part.

Since 1938, our knowledge on the "bipolar differentiation" of eggs and its importance for morphogenesis has made little progress. The nature of the constituents of the egg which separate to different poles and the factors governing this process remain still obscure. However, the observations recorded above and the discussions raised by them have evoked quite a number of researches on the composition of the egg cytoplasm studied by means of cytochemical methods.

These investigations have yielded a great number of data, from which, however, only few general conclusions can be drawn. In general, the results vary greatly; whereas in some forms clear localizations of a special substance may be observed, in other, often nearly related species, nothing of the kind can be detected. For instance, in *Aplysia* the ascorbic acid (vitamin C) presents a particular localization (RIES 1937). In the immature egg it is evenly distributed throughout the cytoplasm, but later it accumulates in a ring of granules in the periphery of the egg. During cleavage, the vitamin C passes chiefly into the blastomere *CD*, later into *3c* and *3d*. However, in other species the ascorbic acid shows no characteristic localization or is lacking altogether.

A greater importance can be attached to the observations on characteristic localizations of certain cell enzymes. In the Ascidia, the myoplasm is very rich in benzidine peroxidase, indophenol oxidase and methylene blue oxidoreducase (RIES 1937; REVERBERI and PITOTTI 1939). The morphogenetic significance of these substances has been shown experimentally. After extirpation of the blastomeres containing the myoplasm, larvae are obtained without muscle cells and in which the above-mentioned reactions remain negative. When the eggs are centrifuged, the displacement of cytoplasmic constituents leads to abnormal larvae showing a chaotic arrangement of the organs; however, in all cases the muscle cells develop from those parts which give the oxidase reactions (RIES 1939). Halves of immature eggs, each containing a part of the indophenol oxidase, can develop to a normal larva. After meridional section of a fertilized egg, each fragment contains the enzyme and develops normally. After equatorial section, only the vegetative half gives the reaction; this half exhibits a bilateral cleavage like the whole egg, whereas the animal half shows an abnormal, radial cleavage (REVERBERI and PITOTTI 1939).

REVERBERI and PITOTTI (1940) studied the distribution of oxidases and peroxidases in various "mosaic" eggs. In *Nereis dumerilii* these enzymes get at cleavage into the blastomere *D*, then the benzidine peroxidase passes especially to the first somatoblast, whereas the indophenol oxidase gets into the second somatoblast. In the Ctenophore *Eucharis multicornis* the peroxidases correspond in their localization to a green fluorescent pigment discovered by SPEK in *Beroë ovata* and which, according to this author, should play an important part in morphogenesis.

Additional observations have been made by PITOTTI (1947). In *Myzostoma glabrum*, the indophenol oxidase reaction in fertilized eggs is restricted to the vegetative pole plasm, which passes at cleavage into the blastomeres 2*d* and 4*D*. *Beroë forskalii* agrees with *Eucharis multicornis* in the distribution of oxidase and peroxidase, but in *Beroë ovata*, which contains the green substance mentioned above, both reactions remain negative. In the eggs of an unidentified species of *Nereis* both reactions are entirely diffuse.

Finally, in the eggs of *Tubifex* the substance of the polar plasms, the great significance of which for the morphogenesis of the embryo has resulted clearly from the researches of PENNERS, gives an elective indophenol oxidase reaction (LEHMANN 1941, 1948).

It seems, therefore, that in many cases special plasms in the eggs, which play an important part in morphogenesis, are characterized by a particularly great amount of oxidative enzymes. On the other hand, it must be emphasized that in other cases with the same methods no definite localization of these substances can be observed in eggs of the "mosaic" type, e.g. in the Polychaetes *Chaetopterus*, *Pomatoceros* (RIES

1937) and *Hydroides* (REVERBERI and PITOTTI 1940) and in the Mollusk *Limnaea* (RAVEN 1945, 1946). Furthermore, even species belonging to the same genus may behave quite differently in this respect (*Nereis*, *Beroë*: PITOTTI 1947).

Ribonucleic acid is another substance, which has been claimed to play an extremely important part in morphogenesis. Its characteristic localization during the development of the amphibian egg has been discovered by BRACHET (1942). Later, this author has adduced a great body of evidence for the thesis that it is of paramount importance in the process of induction. Further investigations of the Brussels School have shown characteristic localizations of ribonucleic acid to occur also in the eggs of other groups: e.g. Mammals (DALCQ & SEATON-JONES 1949), Nematodes (PASTEELS 1948) and insects (MULNARD). On the other hand, no instances are known up to this moment of a particular localization of ribonucleic acid in the "mosaic" eggs of *Spiralia* or *Ascidia*.

Though the investigations of the past 15 years have yielded a number of important facts, it will be evident from this survey that they do not yet permit a definite conclusion on the nature of morphogenetic plasms in general. The whole picture they offer is still rather fragmentary, and our knowledge of the cytochemistry of development will have to be extended much more before a definite conclusion can be drawn. Especially a cytochemical analysis of those eggs that contain plasms of known morphogenetic significance is urgently needed.

The egg of *Sabellaria* offers great advantages in this respect. Its chemical composition, cellular physiology and early history are well known from the classical investigations of FAURÉ-FREMIET (1924). It contains a vegetative pole plasm which rounds off and separates from the egg three times during cleavage in form of a clear-cut antipolar lobe. The causality of its development, and especially the significance of the antipolar lobe for the determination of the parts of the embryo, are well known from the investigations of HATT and NOVIKOFF.

HATT (1931) studied the development of eggs of *Sabellaria alveolata* fused at the uncleaved stage after the artificial dissolution of the vitelline membrane. The products of fusion gave rise to double or multiple embryos, in which the parts produced by each of the components were clearly distinguishable. In no case a single harmonious embryo resulted from fused eggs.

In the next year, HATT (1932) studied the development of isolated blastomeres and of eggs from which an antipolar lobe had been removed. After removal of the first antipolar lobe, the eggs developed to trochophore larvae in which the apical tuft and the posttrochal region of the body were lacking. Similar larvae developed from isolated *AB*-blastomeres. On the contrary, the blastomere *CD*, which contains the substance of the first antipolar lobe, after isolation developed to a fully

normal larva. When the antipolar lobe was removed at the second cleavage, the resulting larvae possessed an apical tuft, but the posttrochal region was lacking. Isolated *C*-blastomeres formed larvae with an apical tuft. An egg, in which $\frac{1}{3}$ of the cytoplasm on the vegetative side had been removed during the first maturation division, yielded a larva which possessed both apical tuft and posttrochal region. The author concludes from his experiments that the substance of the first antipolar lobe contains the determining factors for the formation of the apical tuft and the posttrochal region; the second lobe contains only the latter factors, as the determining factors of the apical tuft have passed in the meantime to blastomere *C*. The size differences between the blastomeres in normal development are only due to the differential distribution of the polar plasm substance. Except a slight difference in the later distribution of the apical tuft factors, these results agree with those found in the egg of *Dentalium* by WILSON (1904).

NOVIKOFF (1938a) studied the development of exogastrulating embryos in *Sabellaria vulgaris*. Both the ectoderm and the endoderm of exogastrulae exhibit a complete self-differentiation corresponding in all details to that found in normal embryos. This proves that the normal spatial relations between the parts of the embryo are not essential for their development; evidently, inductive actions between various organs play no part in the development of this form.

In a second paper (1938b) NOVIKOFF describes the results of isolations of cleavage cells and extirpations of the antipolar lobe in *Sabellaria vulgaris*. They agree with the observations of HATT. The apical tuft develops in dependence of the first antipolar lobe and the *C*-blastomere; the determining factors of the posttrochal region are localized in the first, second and third lobe, finally in the *1D*-blastomere; the short apical cilia, which later replace the apical tuft, are dependent on the blastomeres *A* and *B*. Isolated lobes show rhythmic form changes about synchronously with the rhythm of cleavages, although with some delay. Antipolar lobes grafted into other embryos do not influence their development, but are thrown off. Grafted blastomeres yield the same structures as after isolation; no mutual induction or regulative phenomena occur. It is concluded that the substance of the antipolar lobe cannot be compared to an organizer, which acts by contact on neighbouring cells; it only determines the fate of the cells into which it gets at cleavage.

This conclusion is corroborated by the results of further experiments (NOVIKOFF 1940). By treatment with KCl, cleavage can be temporarily suppressed in *Sabellaria vulgaris*; after return to normal sea water, cleavage recommences. When the eggs are treated before 1st cleavage, many duplicitas embryos are formed. These cleave as 2 *CD*-cells, each having an antipolar lobe. A prolonged treatment yields larvae without apical tuft and posttrochal region, like those obtained from eggs in which the first antipolar lobe has been removed; these larvae develop

from eggs which do not form lobes during cleavage. Other eggs, in which a lobe is formed, develop to normal embryos. When the eggs are treated at the 2-cell stage, in part of the cases supernumerary bristles and eye-spots are formed. When during 1st cleavage at the moment of maximal separation of the antipolar lobe (so-called trefoil-stage) one of the blastomeres is removed, the lobe fuses with the remaining blastomere which develops to a *CD*-larva. The author draws the following conclusions from his experiments: a cytoplasmic material, present in the first antipolar lobe determines the cell into which it gets to a *CD*-cell. This material is considered both a morphogenetic substance and an organizer. The prospective potency of the cells is wider than their prospective fate. The formation of an antipolar lobe is independent of cleavage, and its size does not depend on cell size, but upon the time at which it is formed.

It is clear that the egg of *Sabellaria* appears to be a very favourable object for a cytochemical analysis. It contains a special plasm of known morphogenetic significance and which is clearly recognizable at least at certain stages of development.

We have tried to answer the following questions:

- 1^o Does a "bipolar differentiation", as revealed by a differential vital staining of different egg poles, play a part in the accumulation of the plasm of the antipolar lobe at the vegetative pole of the egg?
- 2^o Is this plasm characterized by the presence of certain substances which may be made visible by cytological and cytochemical methods?
- 3^o If so, does the segregation of the determining factors for apical tuft and posttrochal region to different cells between first and second cleavage express itself in corresponding changes of visible cytoplasmic composition?
- 4^o Does the formation of the lobe depend on the presence at the vegetative pole of the plasm normally situated at this place?

In the course of our investigation furthermore some additional data on the normal development of *Sabellaria*, especially during advanced stages of embryonic development, have been obtained.

The material has been collected and the vital staining experiments and cytochemical reactions on fresh eggs have been done during our stay at the Biological Station at Roscoff, Finistère, France, in September, 1947. We are greatly indebted to the direction and staff of this Station, especially to Prof. P. DRACH, for their kind hospitality and unfailing assistance.

The cytological and cytochemical observations on sectioned eggs have been made by the senior author; the vital staining experiments and cytochemical reactions on fresh eggs have chiefly been executed by the other authors.

1. Unfertilized eggs.

The processes occurring in unfertilized eggs of *Sabellaria alveolata* have been studied by FAURÉ-FREMIET (1924). At the moment of shedding, the eggs are polyedric and irregular; the vitelline membrane lies close to the egg surface, forming some folds. During the first 5 minutes in sea water, the egg becomes regularly spherical. The germinal vesicle, which was somewhat irregular in shape at first, also becomes spherical. Its volume is somewhat more than $\frac{1}{3}$ of the total egg volume. It contains, besides a nucleolus with a diameter of 5–6 μ , chromatic bodies of about 1–2 μ forming irregular groups.

In the next 10 minutes, the germinal vesicle is thrown into folds and decreases in volume. At the same time, the yolk and fat granules of the cytoplasm withdraw from the surface, leaving a hyaline and highly refringent zone. The vitelline membrane which had begun to elevate in the first 5 minutes, now is removed by 6–12 μ from the surface; fine filaments are stretched between the egg surface and the membrane (the hyaline zone and radial filaments had already been observed by HORST 1881). The hyaline layer disappears gradually and the yolk granules reach the egg surface again. The volume of the germinal vesicle diminishes further; an aster appears in the cytoplasm and compresses the germinal vesicle. The centrosphere divides and a spindle is formed. The nuclear membrane disappears, beginning at the side of the spindle. At the same time, the chromatin granules are arranged in threads, which vary at first in number. Then, these threads move to the spindle equator and give rise to 16 chromosomes. The spindle now moves to the egg surface and places itself perpendicular to the latter, the outer aster fusing with the peripheral cytoplasm. The nucleolus remains visible for some time in the cytoplasm. The yolk granules are distributed homogeneously throughout the rest of the egg. At this stage, which is reached after about 30 minutes, the maturation processes are blocked. The egg can be fertilized 45 minutes after oviposition.

Additional data on the processes accompanying the elevation of the vitelline membrane are given by WATERMAN (1934, 1936) and NOVIKOFF (1939) for *Sabellaria vulgaris*. According to the latter, in this species at oviposition in most eggs one indentation is present which is more constant in character and deeper than the others. This large indentation is directly opposite the animal pole of the egg, and coincides with the former point of attachment of the egg in the ovary. The elevation of the vitelline membrane and rounding off of the ovum begin immediately after shedding and are usually completed within 10 minutes. The membrane is originally smooth, but as it becomes further removed from the egg surface it wrinkles considerably. In the newly-shed egg, the cortical cytoplasm, filled with small refringent spherules, lies close against the membrane. Immediately on contact with sea water, the outermost

spherules disappear, leaving a granule-free, hyaline cytoplasm. As the vitelline membrane rises, strands of the hyaline cytoplasm are pulled out with it, so that the connection between egg and membrane is maintained by numerous radiating filaments of granule-free protoplasm. Many of the deeper cortical spherules move slowly toward the periphery of the egg, become smaller and disappear. This leaves a wide zone of hyaline protoplasm with which the strands are continuous. The distance between membrane and egg becomes much greater by a withdrawal of the egg surface, as the zone of hyaline cytoplasm becomes reduced. The strands are reduced to delicate filaments. As the hyaloplasm decreases in extent, its outer boundary becomes increasingly conspicuous. Finally, the hyaline protoplasm is reduced to the narrow cortex of the mature unfertilized egg.

a. *Cytological observations.*

Unfertilized eggs, fixed immediately after shedding, are still irregular in shape. The central part of the egg is occupied by the big clear germinal vesicle, bordered by a distinct nuclear membrane which is thrown into

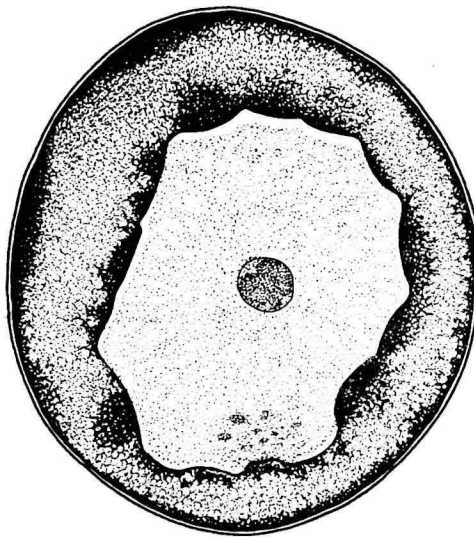


Fig. 1. *Sabellaria*, 10 min. after shedding. UNNA-BRACHET. Ribonucleic acid in peripheral zone of cytoplasm and along nuclear membrane.

folds (fig. 1). In the sections, the nucleoplasm has the appearance of a delicate coagulum; only in places little groups of dark granules are found. It contains a big spherical nucleolus, which has, as a rule, a homogeneous appearance; sometimes, however, in the nucleolus a clear vacuole may be seen. Both the granules in the nucleoplasm and the nucleolus are FEULGEN-negative.

The egg cytoplasm forms a rather narrow layer, 8–10 μ in thickness, surrounding the germinal vesicle. This layer is crowded with spherical

yolk granules, $1.5-2\ \mu$ in diameter; in sections stained with iron haematoxylin, they are nearly black, whereas they are orange-coloured in azan-stained sections. The hyaline zone at this moment has not yet been formed, or is only very thin; the yolk granules nearly reach to the egg surface. The fat globules have not been preserved in paraffin sections.

In eggs fixed 10 minutes after shedding, a distinct hyaline zone has formed. When the eggs are treated after UNNA-BRACHET, the cytoplasm and nucleolus are stained by pyronine. Especially the outer hyaline zone of cytoplasm is heavily stained; a still greater intensity of staining is found, however, in irregular patches of cytoplasm bordering the nuclear membrane (fig. 1). The yolk granules are hardly coloured. In control preparations treated with ribonuclease, the cytoplasm is entirely colour-

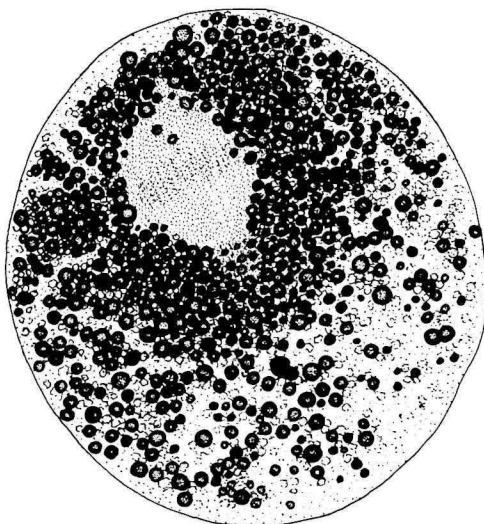


Fig. 2. *Sabellaria*, unfertilized, 80 min. after shedding. CHAMPY, iron haematoxylin. Phosphatide vesicles accumulated around first maturation spindle.

less; the nucleolus is still rather heavily stained, whereas the yolk granules show the same weak staining as before. It may be concluded, therefore, that ribonucleic acids are present on the outside of the nuclear membrane and in the peripheral hyaline protoplasm, to a lesser extent also in the nucleolus. FAURÉ-FREMIET (1924) observed a similar paranuclear localization of basophil substances during oogenesis in *Sabellaria*.

30 Minutes after shedding, part of the eggs still possess an intact germinal vesicle. Contrary to the former stage, now the chromatin bodies in the nucleoplasm are weakly FEULGEN-positive. This reaction increases considerably as soon as the nuclear membrane has disappeared and the chromosomes begin to condense in the spindle equator. Apparently, the accumulation of thymonucleic acid in the chromatin begins already before the dissolution of the germinal vesicle.

80 Minutes after shedding, the eggs possess a first maturation spindle

in metaphase; one of its extremities is attached to the surface, the other is near the centre of the egg. The yolk granules are evenly distributed through the cytoplasm, leaving free the area of spindle and asters and a narrow cortical zone of the cytoplasm. After CHAMPY fixation and staining with saffranin, between the red yolk granules black spherules of similar size are found, which might be fat globules; their distribution corresponds to that of the yolk granules, but they are somewhat less numerous. When the eggs fixed in CHAMPY are stained with HEIDENHAIN's iron haematoxylin, no true mitochondria are visible in the eggs; however, a special kind of spherules is stained in a peculiar manner. They show a black outer margin and a light centre, looking like small rings in the sections. Their size varies very much, from less than $1\ \mu$ to about $4\ \mu$, in contrast to the more uniform sizes of yolk and fat granules. Furthermore, they differ markedly from the latter in their localization, being concentrated around the maturation spindle, which they surround like a dark halo (fig. 2). In earlier stages, with intact germinal vesicle, they show no special accumulation, however. If they are preformed elements and no mere fixation artifacts (myelin figures?), their staining reactions seem to show that they are rich in phosphatides. According to FAURÉ-FREMIET, phosphatides are found in diffuse and colloidal form in the cytoplasm of *Sabellaria* eggs. It is probable, therefore, that an accumulation of phosphatides, either dissolved in the cytoplasm or bound to special granules, around the maturation spindle occurs after the dissolution of the germinal vesicle.

It was tried to demonstrate the so-called Golgi apparatus in the eggs by post-osmication after CHAMPY fixation, but no clear pictures were obtained. Presumably, the conditions of penetration of the fixing fluids into these small eggs are not very favourable for the production of these structures (cf. PALADE and CLAUDE 1949).

b. *Centrifuged eggs.*

FAURÉ-FREMIET (1924) studied centrifuged unfertilized eggs of *Sabellaria alveolata*. The eggs showed three distinct zones. In the middle region, the cytoplasm was found, a hyaline and viscous substance, which was dark in dark-field illumination. At one of the poles a rather considerable mass of feebly refringent heavy granules was formed by the yolk; it has a lilac colour by pigment. At the other pole, the strongly refringent light oil globules are accumulated. By means of vital stains (neutral red, Nile blue or cresyl brilliant blue) a few heavily stained granules can be demonstrated in all parts of the eggs. The maturation spindle has been thrown, as a rule, into the yolk zone.

Also HARRIS (1935) centrifuged unfertilized eggs of *S. alveolata*. Under a force of about 3000 g, 30 sec. of centrifuging was usually sufficient to displace the heavy granules into one hemisphere in "mature" unfertilized eggs. If the eggs were centrifuged before disappearance of the

germinal vesicle, this duration of treatment was not sufficient to move the granules to any appreciable extent. Apparently, the viscosity of the egg is greater before than after the breakdown of the germinal vesicle; for the latter, an approximate value of 0.2 c.g.s. units was computed. The granules in the cytoplasm are in active Brownian movement, but the random character of this movement was masked by very pronounced streaming movements. They bring about a more or less complete mixing of the separated constituents of the egg in a much shorter time than would be the case if Brownian movement were the only factor involved in the redistribution; almost complete mixing occurs in an hour from the time of centrifuging.

We centrifuged unfertilized eggs, about 20 minutes after shedding,

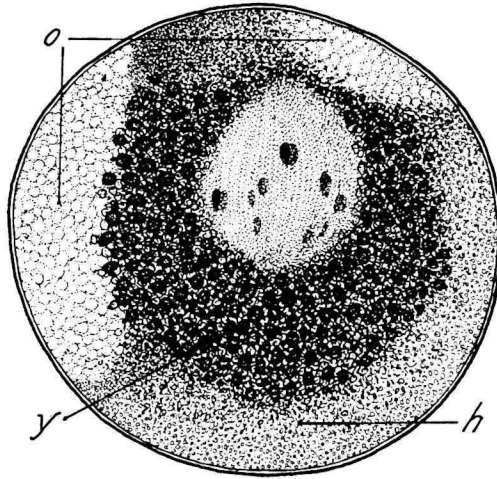


Fig. 3. *Sabellaria*, unfertilized, 40 min. after centrifuging. Redistribution of materials. Yolk (y) accumulated around maturation spindle. o = oil, h = hyaloplasm.

for 5 minutes at about 2500 r.p.m. They showed a distinct stratification. Sections of eggs fixed 13 minutes after centrifuging still show the three zones rather sharply delimited. The oil zone has a vacuolar appearance, the oil being dissolved away in the preparation of the paraffin sections. The hyaloplasm shows a dense, finely granular structure, being violet after haematoxylin-eosin and orange-yellow after azan staining; along the boundary with the oil and yolk zones there is a somewhat denser, more deeply stained band. In some eggs, in azan-stained sections a single layer of rather big blue granules, which may be lying in a vacuole, is found at the boundary of hyaloplasm and oil zone. The yolk zone contains the densely-packed yolk granules. Their staining properties, in these eggs, differ somewhat from those in uncentrifuged eggs; after iron haematoxylin-eosin staining, they are brownish red, except the most peripheral granules which are darker till nearly black; in azan-stained sections, the colouration of the yolk granules is a purplish brown. It may be that under natural circumstances the proteid granules of the egg are coated,

e.g. by a thin layer of lipids, which has been separated and thrown centripetally by centrifuging, and that this accounts for the change in staining properties observed. Contrary to the statement of FAURÉ-FREMIET, the maturation spindle at this moment is always found in the hyaloplasm zone, but usually in the region adjoining the yolk zone; often, one of the ends of the spindle has sunken somewhat into the latter, or the whole spindle indents the boundary between hyaloplasm and yolk.

40 Minutes after centrifuging, the stratification is much less distinct. The oil and yolk zones have extended by a redispersion of the granules, the hyaloplasm zone has become very narrow or has entirely disappeared. The yolk granules, in their redistribution, accumulate around the maturation spindle; in nearly all eggs, the latter is entirely surrounded by a dense mass of yolk granules (fig. 3). This observation points to an attraction exerted by the spindle upon the yolk granules in these eggs.

In eggs fixed 85, 111 and 144 minutes after centrifuging, the same relations have been found. The yolk remains accumulated around the spindle. Apparently, no further changes in these eggs occur until degenerative phenomena begin. As a matter of fact, already 111 minutes after centrifuging, the spindles are becoming less distinctly visible; evidently, degenerative changes are already beginning.

c. *Vital staining.*

With neutral red and nile blue hydrochloride, the unfertilized eggs are stained homogeneously in red resp. blue; only the outer hyaline layer remains lighter or colourless. Presumably, this colouration is due to staining of the yolk granules (cf. FAURÉ-FREMIET 1924).

When newly-shed eggs are put in a weak solution of brilliant cresyl violet, they take a weak violet colour. In centrifuged eggs the hyaloplasm remains colourless; both the oil zone and the yolk zone are stained; at the margin of oil and plasm zone there is a narrow layer of pink granules.

In Janus green, the eggs remain colourless while alive; as soon as they begin to cytolys, they become intensely blue-green.

d. *Cytochemical reactions.*

The Nadi-reaction, for the detection of indophenol oxidase, in all its modifications, and the benzdine peroxidase reaction remained entirely negative; this agrees with the observations of FAURÉ-FREMIET. On the contrary, the reaction with sodium nitroprussiate for the detection of glutathione, which gave no good results to FAURÉ-FREMIET, proved to be positive in our experiments. As a matter of fact, the already difficult reaction becomes less easy still by the presence of the natural pigment of the eggs, but after some experience the momentary colour flash due to the presence of sulphhydryl groups can be distinguished from the simultaneous change of colour of the egg pigment under the action of ammonia.

When the reaction is executed with fresh eggs, the germinal vesicle gives a weak positive reaction; after repeated treatment with a 10 % trichloroacetic acid solution there is a distinct reaction of the central part of the egg, which extends towards the periphery, but fades away before reaching the surface. In fresh centrifuged eggs, the reaction is likewise confined to the germinal vesicle; in centrifuged eggs treated with trichloroacetic acid a distinct reaction occurs, beginning in the hyaloplasm zone but extending secondarily into the adjoining part of the yolk zone. It can be concluded from these observations that the germinal vesicle contains free glutathione in its reduced form, whereas bound sulphydril compounds are found also in the cytoplasm surrounding the germinal vesicle.

No positive results were obtained with the vitamin-C reaction after GIROUD and LEBLOND.

After treatment with a iodine solution, the eggs show a homogeneous brownish red staining; control eggs treated with saliva prove that this staining is due to glycogen. In centrifuged eggs, the glycogen reaction is restricted to the hyaloplasm. This agrees with the observations of FAURÉ-FREMIET.

In centrifuged unfertilized eggs treated with Sudan III, both the oil and the yolk zone are stained with nearly equal intensity; the hyaloplasm shows a very weak colouration. Evidently, the yolk granules contain, besides proteins, a certain amount of lipid substances. It was tried to determine the nature of the lipid compounds of the oil zone, using the dichotomous table of LISON (1936, p. 214). The fatty substances had no own yellow or brown colour. The reaction of LIEBERMANN according to the technique of ROMIEU was negative. Under the polarization microscope between crossed nicols the fat zone showed no polarization cross. The reaction of SMITH-DIETRICH was positive, causing a distinct blackening of the oil cap. These reactions prove the presence of lipins in the oil zone of the centrifuged egg. As, on the other hand, the reaction of L. SMITH gave a distinct red staining with Nile blue sulphate, also unsaturated glycerides seem to be present. These results are in good agreement with the quantitative and cytochemical determinations of FAURÉ-FREMIET (1924).

Finally, with the xanthoprotein reaction centrifuged unfertilized eggs showed a yellow colouration both of the hyaloplasm and the yolk zone; in the hyaloplasm it was stronger than in the yolk.

2. Fertilization and maturation.

Observations on fertilization and maturation in *Sabellaria* have been made by HORST 1881, FAURÉ-FREMIET 1924, WATERMAN 1934, and NOVIKOFF 1939.

According to FAURÉ-FREMIET, the egg of *Sabellaria alveolata* can be

fertilized from the moment that the first maturation spindle has reached the metaphase; the egg remains fertilizable for more than an hour; afterwards, the results of fertilization are uncertain. The penetration of the sperms takes place in 3—4 minutes. The sperm head pushes against the vitelline membrane which is somewhat indented. Nearly immediately a hyaline protuberance appears on the egg surface, and protrudes like a pseudopodium towards the sperm which now pierces the membrane. This fertilization cone may take various shapes. When the sperm head has penetrated into the fertilization cone, the latter withdraws, and only a lenticular spot of hyaline protoplasm indicates the point of penetration.

Immediately after insemination, the first maturation division continues. At the anaphase of this division, the outer end of the spindle rises as a pointed hyaline cone above the egg surface, which grows during telophase, while the egg is markedly flattened along the spindle axis. Then a rapid constriction isolates the first polar body, about 15 minutes after fertilization at 18° C. At this moment, the egg surface is lifted for some moments into short hyaline lobules.

During metaphase of the second maturation division the eggs become spherical, but about 17 minutes after the formation of the first polar body the egg flattens again and the outer end of the spindle protrudes once more above the surface. While the second polar body is extruded quickly like the first, the opposite side of the egg swells slightly, so that the egg becomes top-shaped. This stage precedes immediately the fusion of the pronuclei and the formation of the first cleavage spindle, the axis of which is exactly perpendicular to that of the maturation spindles.

Cytological observations show that the chromosomes of the first maturation division at metaphase have the shape of long flexuous filaments; later, they present an annular configuration. At anaphase the two daughter-plates each contain the diploid number of 16 chromosomes. When the polar body is extruded, the spindle contracts to about $\frac{1}{4}$ its former length. The chromosomes of the egg are thereby drawn towards the animal pole; they do not form a nucleus, but remain heaped together. The sperm nucleus at first shows no change in shape or volume, but at the telophase of the first maturation division it swells slightly.

When the remains of the first spindle have disappeared, the chromosomes come apart; in the meantime, they have fused in pairs to 8 dyades. These arrange themselves into the equatorial plate of the second maturation spindle. The latter is short (about $\frac{3}{4}$ the length of the first maturation spindle), biconical in shape and oblique with respect to the surface; then, it approaches the latter and the astral rays extend. At anaphase 8 single chromosomes go to each end of the spindle. At telophase, the chromosomes swell into 8 vesicles, which soon coalesce into an irregular ♀ pronucleus; later, this becomes spherical.

The sperm nucleus swells too; at first, it shows some chromatin masses,

then it becomes vesicular and forms the ♂ pronucleus. The pronuclei approach each other; they are surrounded by temporary cytoplasmic radiations. They can either coalesce altogether or remain separated.

WATERMAN 1934 and NOVIKOFF 1939 give additional data on fertilization in *Sabellaria vulgaris*. During penetration of the sperm the protoplasmic strands connecting the egg surface with the vitelline membrane are withdrawn into the egg. Penetration may take place at any point around the periphery of the egg, but this occurs more frequently in the animal hemisphere. The fertilization cone may contain, besides hyaline protoplasm, also granular endoplasm in its centre; this depends, according to NOVIKOFF, on the age of the egg at fertilization. The head and the mid-piece of the sperm separate from the tail and slip through the vitelline membrane; the tail remains outside and regains its motility for some time. Sometimes the first polar body appears before the sperm has actually entered.

a. *Cytological observations.*

Eggs of *Sabellaria alveolata* were fertilized 20 min. after shedding and fixed 7 min. after insemination. In some of these eggs the germinal

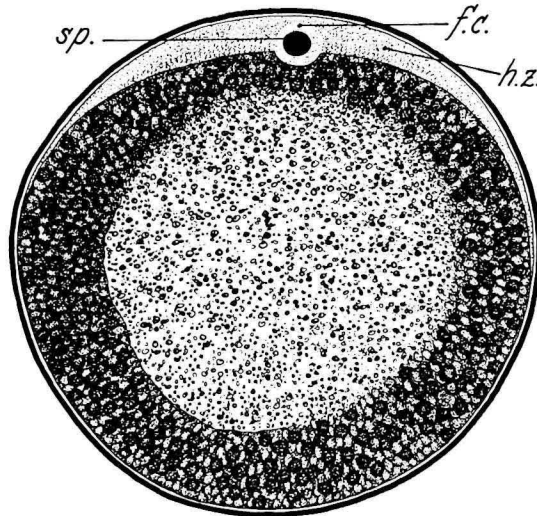


Fig. 4. *Sabellaria*, 7 min. after insemination. Beginning dissolution of germinal vesicle. Sperm head (sp.) in fertilization cone (f.c.). Thickening of hyaline zone (h.z.) near fertilization cone.

vesicle is still intact; in other eggs, its membrane has partly or entirely been dissolved and the central part of the egg consists of a clear yolk-free area, in which the maturation spindle is forming.

Insemination has already taken place. In nearly all eggs a sperm head is present, lying subcortically in the fertilization cone. This forms a local thickening of the outer hyaline zone of the egg, protruding outwards as a slight bulge of the egg surface, and inwards towards the yolk-filled

deeper layer of the egg (fig. 4). Moreover, the hyaline zone shows an unequal thickness, being thickest near the fertilization cone and narrowing gradually towards the opposite side of the egg.

It must be emphasized that a sperm has entered even in eggs with intact germinal vesicle; evidently, the eggs are fertilizable even before the breakdown of the germinal vesicle. As soon as the sperm head has entered the egg, it begins to swell; whereas sperm heads situated on the outside of the membrane have, in the sections, a diameter of $2.4\ \mu$, those within the egg have diameters up to $4.0\ \mu$. Moreover, the biggest ones are already surrounded by a vacuolar space; evidently, fluid has accumulated around the sperm nuclei. The latter at this stage are only weakly FEULGEN-positive, like the chromosomes which are forming in the maturation spindle.

UNNA-BRACHET preparations show that the nuclear membrane nucleotides, surrounding the germinal vesicle, remain visible for some time after the breakdown of the latter, as a dark ring around the nuclear area. The asters and maturation spindle are distinctly coloured. Moreover, the hyaline outer plasm zone is rich in ribonucleic acid, but the staining is somewhat weaker in the neighbourhood of the entrance point of the sperm.

15 Minutes after insemination, the sperm nuclei have swollen still more; their diameter amounts to $5-6\ \mu$. They show a somewhat uneven, granular surface and are situated in a distinct vacuole about $8-9\ \mu$ in diameter. The maturation spindle in metaphase in some cases has reached the surface with one of its extremities; here an accumulation of hyaline yolk-free plasm has been formed. The yolk granules have invaded the nuclear area and in some cases are already evenly distributed through the egg, leaving free only the spindle and asters and the outer hyaline plasm zone. On the contrary, the "phosphatide vesicles", stained by HEIDENHAIN's iron haematoxylin after CHAMPY fixation, show a marked concentration around the maturation spindle.

22 Minutes after insemination, the first maturation spindle is in early anaphase. The sperm nucleus, still situated in its vacuole, has again slightly increased in size, reaching a diameter of 5 by $7\ \mu$. It shows no normal nuclear structure, being homogeneously stained, but its surface is still uneven and granulated. In FEULGEN preparations, the interior of the sperm head is weakly FEULGEN-positive, but the superficial granules are intensely stained. Evidently, they represent chromatin bodies of the nucleus. The chromosomes of the early maturation spindle show a peculiar appearance; they are threadlike with little FEULGEN-positive thickenings at regular distances (fig. 5). In UNNA-BRACHET preparations, the sperm head appears to be most pyroninophil of all structures, followed immediately by the maturation spindle; the outer hyaline plasm stains with much less intensity.

The next stage is formed by eggs fixed 55 min. after insemination.

Development has progressed considerably. The first polar body has been formed; the second maturation spindle in metaphase is situated at the animal pole with its axis perpendicular to the surface. The yolk fills the remaining part of the egg. The outer hyaline plasm has been reduced to a narrow layer. The sperm nucleus was not visible at this stage.

77 Min. after insemination both polar bodies have been formed. In part of the eggs the two pronuclei are lying against each other near the centre of the egg; in other eggs a cleavage spindle in metaphase has been formed. The yolk is evenly distributed through the cytoplasm. At the

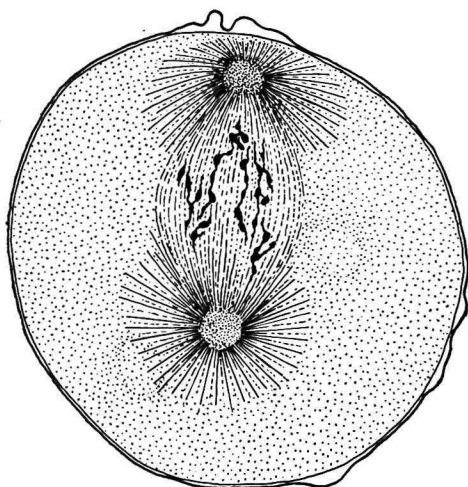


Fig. 5. *Sabellaria*, 25 min. after insemination. FEULGEN. First maturation spindle, pro-metaphase.

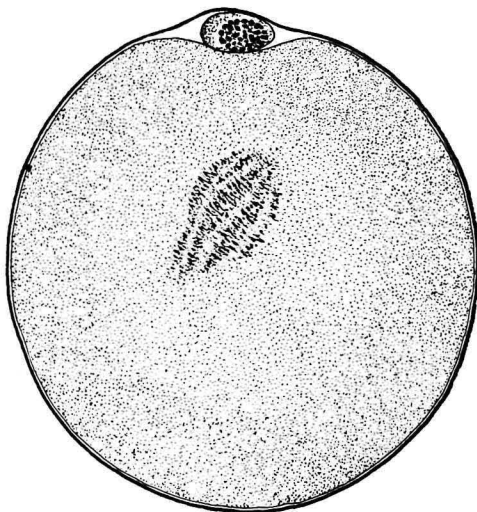


Fig. 6. *Sabellaria*, 77 min. after insemination. FEULGEN. Prophase of first cleavage mitosis.

vegetative side the surface of the egg is slightly bulged. In some cases, the staining of the yolk in this part seems to differ slightly from that in the rest of the egg; this difference is very small, however. In FEULGEN preparations the chromosomes of the polar bodies are deeply stained. The pronuclei show a scarcely perceptible diffuse staining. The prophase nuclei contain delicate chromatin threads, forming large spirals and in which dark granules (chromomeres) are lying (fig. 6). After UNNA-BRACHET staining, the subcortical hyaline plasm is distinctly coloured. The nucleoplasm of the pronuclei is colourless, but their nuclear membranes are distinctly stained. The cleavage spindle is heavily stained.

b. *Centrifuged eggs.*

Eggs were inseminated about 45 min. after shedding and centrifuged 6 min. later for 5 min. at about 2500 r.p.m. Unfortunately, most eggs of this batch proved to be unfertilized so only a few data on centrifuged fertilized eggs could be obtained.

9 Min. after centrifuging, the eggs are still fully stratified. The stratification of the fertilized eggs agrees with that of the unfertilized ones. In the oil zone, a fine plasm reticulum between the oil vacuoles exists, with small black granules lying in the points of junction. In the fertilized eggs, both polar bodies have been formed; in one case, they are lying near the boundary of oil and plasm zone, about 45° from the centripetal pole of the egg; in another case, they have a similar localization, but are situated about 60° from the centripetal pole. Big polymorphic pronuclei, which appear as if they have been formed by the fusion of several karyomeres, are lying near each other in the plasm zone. Small asters are found near them.

41 Min. after centrifuging, the redispersion of the oil and yolk have begun. The zone of hyaloplasm is only narrow and not sharply delimited towards the other zones. In one egg, a cleavage spindle in metaphase is formed, lying obliquely at the margin of the hyaloplasm and yolk zones, one of its ends being entirely surrounded by yolk granules.

Later stages of these centrifuged eggs will be described below.

c. *Vital staining.*

In neutral red and Nile blue hydrochloride, the staining of fertilized eggs agrees with that of unfertilized ones. The outer hyaline layer remains lighter than the inner part of the egg; at the animal side this layer is broader. In centrifuged eggs stained with neutral red, the yolk zone stains deep-red; the hyaloplasm takes an orange-brown colour, beginning near the oil zone but extending gradually towards the margin of the yolk zone. This differential staining of hyaloplasm and yolk agrees with the observations of FAURÉ-FREMIET (1924).

In brilliant cresyl violet, the eggs are evenly stained immediately after fertilization; when the maturation divisions begin, the polar area at the animal pole becomes colourless. After the extrusion of the second polar body, this clear polar area disappears. The staining of centrifuged eggs agrees with that of the unfertilized eggs.

Neither Janus green nor methylene blue stain intact eggs.

d. *Cytochemical reactions.*

Also in fertilized eggs, the Nadi reaction and the benzidine peroxidase reaction were negative.

With sodium nitroprussiate, fresh fertilized eggs gave a very weak reaction of the whole cytoplasm, which is most intense in the central part of the egg. After treatment with 10 % trichloroacetic acid, a distinct reaction in the central part of the egg occurred. Fresh centrifuged eggs showed a weak reaction in the upper part of the yolk zone and the adjacent region of the hyaloplasm; centrifuged eggs treated with trichloroacetic acid gave an intense reaction, beginning in the central part

of the hyaloplasm zone and extending peripherally with decreasing intensity.

The vitamin-C reaction after GIROUD and LEBLOND yielded only negative results.

In centrifuged fertilized eggs treated with Sudan III, the staining of the yolk zone has diminished as compared with unfertilized eggs; now the oil zone shows a much deeper staining than the yolk zone. With the reaction of L. SMITH, the oil zone gave a distinct red staining with Nile blue sulphate.

3. Cleavage and gastrulation.

Cleavage in *Sabellaria* has been described by FAURÉ-FREMIET (1924), HATT (1932) and NOVIKOFF (1938b).

According to FAURÉ-FREMIET, in the egg of *Sabellaria alveolata*, after the extrusion of the second polar body, the top-shape of the egg becomes still more pronounced by the formation of a protoplasmic gibbosity opposite the flattened polar side; this is called the cordiform stage. In 5–6 minutes the antipolar gibbosity transforms into a clearly defined lobe, 25–30 μ in diameter, while the egg elongates markedly perpendicular to the axis of the maturation spindle. Afterwards, the lobe fuses with the egg and the shape of the egg becomes cordiform again; then, the lobe reappears. About 40 minutes after the extrusion of the second polar body, the egg elongates transversely by the lengthening of the cleavage spindle. The latter, which is perpendicular to the axis of the maturation divisions, is at first somewhat shorter than the first maturation spindle. At the end of prophase, after the disappearance of the nuclear membrane, two groups of chromosomes can be observed in the spindle, which correspond to the pronuclei. The chromosomes are arranged in the equatorial plate and divide longitudinally; at anaphase each daughter plate consists of 16 chromosomes. The lengthening of the spindle causes the transversal elongation of the egg at metaphase. The gelification of the asters extends towards the periphery in the animal $\frac{2}{3}$ of the egg only; when the asters contract, a flattening of the egg occurs. The cytoplasm of the vegetative side of the egg does not take part in these transformations and forms a semifluid mass which by an annular furrow is separated more and more from the division zone. At anaphase, when a meridional furrow separates the two first blastomeres, the antipolar lobe is entirely independent and contiguous with the rest of the egg ("trefoil-stage"). At this moment, the egg surface shows fluctuating movements. The asters of the first cleavage mitosis are equal in size, and the two first blastomeres have about the same volume. At telophase, during the reconstitution of the nuclei, an asymmetry becomes visible, however; one of the asters disappears earlier than the other. The antipolar lobe fuses with the blastomere where the disappearance of the aster

is slightly ahead; so a 2-cell stage with two unequal cells is formed. These cells get for some moments a nearly spherical shape, then they are elongated at the metaphase of the second cleavage division in two slightly different directions. The two spindles of this division are lying in planes parallel to the first cleavage plane, but their axes form an angle of about 45° . They are situated again in the animal half of the egg. During the division of the macromere *CD*, one observes again the temporary formation of an antipolar lobe. The 4-cell stage is formed 15–20 minutes after the 2-cell stage. From this stage on, the division of the blastomeres is asynchronous, a 6-cell stage precedes the 8-cell stage.

HATT (1932) gives some additional observations. The antipolar lobe at the trefoil-stage, viewed from the side, seems to have the same size as the blastomeres. In reality, the latter are somewhat elongated in a perpendicular direction, so that the antipolar lobe is smaller. The second lobe seems to be smaller than the first; part of the substance of the latter should, therefore, remain in blastomere *C*, which is intermediary in size between *A* and *B*, on one hand, and *D*, on the other.

NOVIKOFF (1938*b*) records the relations in *Sabellaria vulgaris*. Cleavage begins, at room temperatures, approximately 20 min. after the extrusion of the second polar body. The antipolar lobe at first cleavage, when viewed from the vegetal pole, is seen to be considerably smaller than the first two blastomeres. The visible constituents of the lobe cytoplasm do not differ from those of either blastomere, except that there is no spindle area in the lobe. About 15 min. after it first appears, the lobe flows into one of the blastomeres. The second polar lobe forms in the *CD*-blastomere only, and is smaller than the first lobe. When it flows back into one of the daughter cells at the completion of the division, the four quarter-blastomeres consist of two equal-sized cells, *A* and *B*, the products of the division of *AB*, a slightly larger cell, *C*, and a much larger cell, *D*. During the next division, when the micromeres are produced, a third polar lobe, formed from the *D*-cell, flows into the *D*-macromere, 1*D*. This lobe is smaller than the second lobe and is more variable than the preceding lobes; in many cases this lobe does not become distinctly separated from the dividing *D*-cell.

a. *Cytological observations.*

In a batch fixed 1 h. 32 min. after fertilization, various stages of first cleavage mitosis are present; metaphase, early and late anaphase, telophase and 2-cell stages with reconstituted nuclei. At telophase, the chromosomes in both blastomeres swell into little vesicles; these karyomeres fuse to a polymorphic resting nucleus. Metaphase and early anaphase eggs are clearly "top-shaped"; at a mid-anaphase stage, the protoplasmic gibbosity at the vegetative pole transforms into a clearly defined antipolar lobe, which remains separated during late anaphase and telophase stages and fuses with one of the blastomeres when the

karyomeres begin to coalesce into a polymorphic nucleus. Then, there is a clear size difference between the blastomeres *AB* and *CD*. In all stages the yolk granules are filling the greater part of the cytoplasm, leaving free the cleavage amphiasters and nuclei. The contents of the antipolar lobe do not differ from the rest of the peripheral cytoplasm; no differences in density or staining properties of the yolk can be observed. In FEULGEN preparations, the chromosomes of the polar bodies form compact heavily-stained masses. The metaphase, anaphase and telophase chromosomes are moderately stained, whereas the karyomeres of the reconstituting nuclei are only weakly and diffusely FEULGEN-positive. In UNNA-BRACHET preparations the early karyomeres are embedded in a deeply-staining mass, representing the remnant of the aster; the subcortical hyaline plasm is moderately stained.

The eggs of a batch fixed 1 h. 55 min. after fertilization vary from a 2-cell stage with early reconstitution nucleus to the anaphase of the next cleavage mitosis. The polymorphic resting nuclei show a further swelling, during which they round off to big vesicular nuclei of nearly spherical shape. The second cleavage mitosis seems to begin, on an average, somewhat earlier in *CD*; also at later stages mitosis in *CD* is slightly ahead of that in *AB* (fig. 7) (this is also shown in the figures of FAURÉ-FREMIET's paper). The yolk granules are still evenly distributed through the egg, but the "phosphatide vesicles", stained by HEIDENHAIN's iron haematoxylin after CHAMPY fixation, are concentrated around the nuclei and cleavage spindles as they were at earlier stages around the maturation spindle. In FEULGEN preparations the resting nuclei show scarcely any staining. At beginning prophase, delicate coiled threads appear which are weakly FEULGEN-positive. As the nuclear membrane disappears and the chromosomes arrange themselves into the equatorial plate, their staining increases and with the completion of spiralization the metaphase chromosomes have become short deeply-stained rods (fig. 7).

In UNNA-BRACHET preparations, the karyoplasm of the resting nuclei stains green with methyl green, but the nuclear membrane shows a strong pyroninophily. The yolk granules are colourless, the cytoplasm between them is distinctly stained with pyronine; the subcortical hyaline layer shows a somewhat deeper staining. The cleavage spindles are deeply stained with pyronine; the chromosomes are green. The polar bodies show an intense pyroninophily.

At 2 h. 2 min. after fertilization late anaphase and telophase stages of the 2nd cleavage mitosis have appeared. A well-developed antipolar lobe has formed at *CD*, which fuses with *D* during telophase. Its cytoplasm agrees with the rest of the peripheral plasm in its composition. When the telophase chromosomes begin to swell into karyomeres, at the same time their FEULGEN-reaction diminishes considerably. The pycnotic nuclei of the polar bodies show a very strong FEULGEN-staining.

The reconstitution of the nuclei of the 4-cell stage can be studied at

2 h. 15 min. after fertilization. As before, the karyomeres after swelling coalesce into a polymorphic nucleus, which rounds off with further swelling into a resting nucleus. In FEULGEN preparations, the progressive decrease of colouration during this phase, from the heavily-stained anaphase chromosomes to the scarcely perceptible staining of a delicate reticulum in the resting nuclei, can be followed step by step. In UNNA-BRACHET preparations, the resting nuclei of the 4-cell stage, like those of the previous stage, show a distinct accumulation of nuclear membrane

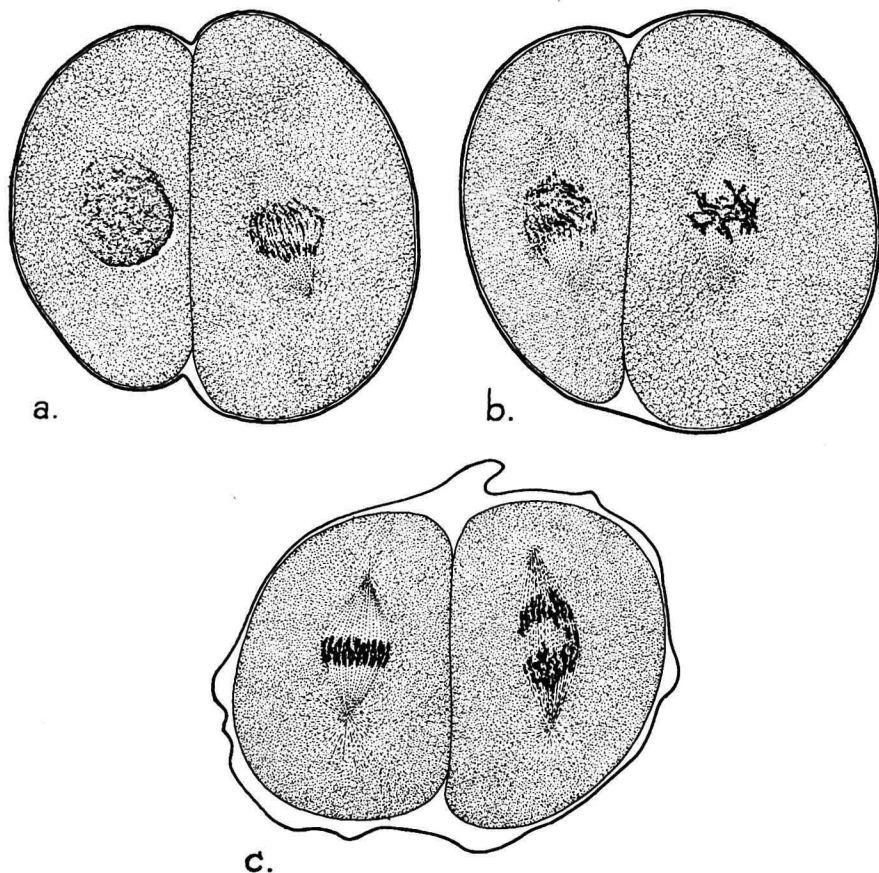


Fig. 7. *Sabellaria*, 1 h. 55 min. after insemination. FEULGEN. Pro- to anaphase of second cleavage mitosis.

nucleotides; their karyoplasm stains with methyl green. The peripheral layer of the cytoplasm is more strongly basophil than the central part. At this stage, the density of the yolk seems to become somewhat greater on the vegetative than on the animal side of the blastomeres, but the difference is only slight.

The 3rd cleavage mitosis was studied in the eggs of another batch, fertilized 35 min. after shedding and fixed 2 h. 5 min. after fertilization. The cleavage spindles of this division have turned into a more vertical

position. A third antipolar lobe is formed temporarily at *D*; the density of the yolk in this lobe is somewhat greater than in the rest of the egg. In general, however, the distribution of the yolk is still very homogeneous. The same holds true with regard to the fat globules, visible as black spherules after CHAMPY fixation and staining of the sections with saffranin. On the contrary, the "phosphatide vesicles" still form dense haloes around the nuclei and spindles.

At 2 h. 45 min. the eggs of this batch show the formation of the 2nd micromeres. The synchronicity of the cleavage divisions has been lost; part of the blastomeres show a polymorphic or vesicular nucleus, whereas others possess a cleavage spindle. In the vesicular nuclei for the first time a distinct nucleolus may be found. The "phosphatide vesicles" show the same arrangement as before, each nucleus and spindle being surrounded by a dark circle of vesicles in CHAMPY-fixed eggs stained with HEIDENHAIN'S iron haematoxylin. In the centre of the egg a small

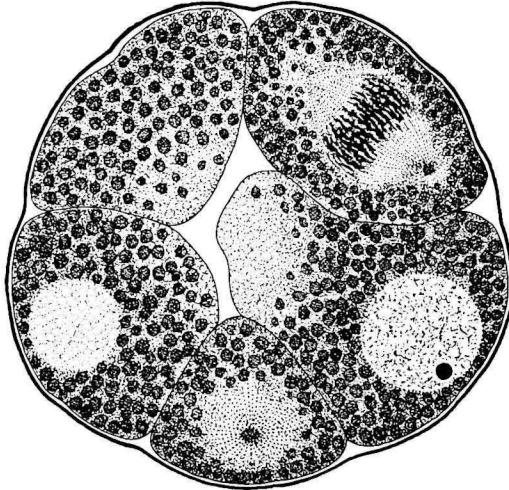


Fig. 8. *Sabellaria*, 2 h. 45 min. after insemination; 8—12 cell stage. Formation of cleavage cavity.

cleavage cavity has been formed between the cells. One of the macromeres protrudes with a conical protuberance, consisting of hyaline protoplasm with scattered yolk granules, into this cavity (fig. 8).

About $4\frac{1}{2}$ h. after fertilization the eggs have formed early blastulae with a small cleavage cavity, situated eccentrically near one of the poles. At this stage, the polar bodies, which in previous stages were situated at the animal pole beneath the vitelline membrane, have been incorporated into the egg, where they are found as spherical structures, containing a pycnotic nucleus, between the blastomeres near the cleavage cavity; both polar bodies may lie at some distance apart. FEULGEN preparations show that, contrary to previous stages, now also the resting nuclei are distinctly FEULGEN-positive, though less than the cleavage

chromosomes; with increasing swelling of the nuclei during the resting stage, however, their colourability gradually decreases. The pycnotic nuclei of the polar bodies are strongly FEULGEN-positive. In UNNA-BRACHET preparations, the eggs show a rather uniform red staining, but the ectoplasm is somewhat darker than the endoplasm. The cells in mitosis have a clearly less basophil cytoplasm than the other ones. The chromosomes are green, the resting nuclei greenish with deep red nucleoli and nuclear membranes.

Nine hours after fertilization gastrulation has begun. There is an eccentric cleavage cavity, which is bordered at the animal side by a thin roof of one layer of rather small cells, forming a regular epithelium (fig. 9). The bottom of the cleavage cavity is formed by a thick mass of bigger cells. A small archenteron invagination is entering into this

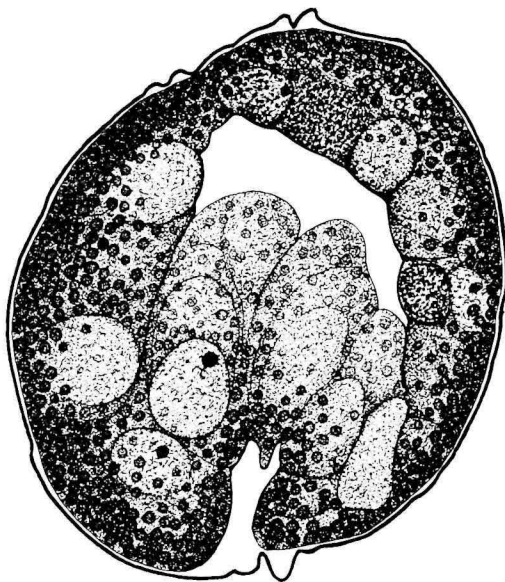


Fig. 9. *Sabellaria*, 9 h. after fertilization. Gastrula.

mass. The polar bodies with pycnotic nuclei are still visible between the cells. The distribution of the yolk granules is a peculiar one; they form a layer of about 2—3 rows of big granules all around the egg beneath the surface and along the archenteron lumen. The interior of the cells contains much less yolk granules. Whereas the yolk granules in the ectoplasm are deep orange-coloured in azan-stained sections, in some of the endoplasmic yolk granules the staining has changed to blue. Presumably, this change in staining properties marks a beginning intracellular digestion of the yolk.

FEULGEN preparations of gastrulae 12 h. after fertilization show that the staining of the nuclei differs very much. The staining of chromosomes in dividing cells is strongest; small resting nuclei are still deeply

stained, whereas, in general, the staining decreases with swelling of the nuclei. However, nuclei of nearly equal size may show considerable differences in staining intensity; this points to a beginning differentiation of the cells at this stage. The pycnotic nuclei of the polar bodies are still strongly FEULGEN-positive.

b. *Centrifuged eggs.*

From a batch, centrifuged 6 minutes after insemination (its previous history has been recorded above, p. 19), a number of eggs were fixed 111 minutes after centrifuging. Only one of the eggs had been fertilized. It is in the 8-cell stage; all blastomeres have polymorphic or vesicular resting nuclei. The substances separated by centrifuging are still clearly recognizable and rather sharply delimited. The cells 1a, 1A, 1b and 1B consist entirely of oil and hyaloplasm; on the contrary, 1c, 1C, 1d and 1D are crowded with densely-packed yolk granules, with the exception of 1D, part of which consists of clear cytoplasm with only some scattered granules. Evidently, at first cleavage the substances had been neatly divided over the blastomeres, CD containing all the yolk and AB consisting entirely of oil and clear cytoplasm.

Another sample of this batch was fixed 144 min. after centrifuging. It contains 3 fertilized eggs, which have reached the stage of 4th cleavage mitosis, part of the 8 cells possessing a cleavage spindle. A clear distinction can be made between those cells, which are filled with yolk granules, and other ones, only containing oil and hyaloplasm; the intracellular boundary between the latter two components has become quite unsharp, however. Yolk-laden and yolk-free cells are arbitrarily distributed, indicating that the axis of centrifugation has made any angle with the polar axis of the eggs. In two of these eggs, one of the polar bodies has become incorporated into a micromere; presumably, this is the beginning of the inward migration of the polar bodies, which are found in the interior of the embryo at later stages.

Another batch of eggs from the same female, fertilized about 45 minutes after shedding, were centrifuged 60 min. after fertilization for 5 minutes at 2500 r.p.m. A sample of these eggs were fixed 10 minutes after centrifuging. It contains one fertilized egg, which is at the 2-cell stage. There is a distinct stratification; the oil zone, hyaloplasm and yolk zone are sharply delimited. The axis of stratification is perpendicular to the polar axis of the egg and nearly parallel to the plane of first cleavage. Hence, both the AB- and the CD-cell contain part of all 3 layers of substances. The boundaries between the layers are only slightly disturbed by the formation of the cleavage plane. In both cells, a prophase nucleus is lying in the hyaloplasm zone. It is flanked by two small asters, and the nuclear membrane is being dissolved at this place.

A sample, fixed 47 min. after centrifuging, contains a 4-cell stage in anaphase of the 3rd cleavage mitosis. The zones of substances are still

clearly distinguishable and have rather sharp boundaries. *C* contains only oil and hyaloplasm, *B* oil, hyaloplasm and yolk, *D* yolk with a narrow border of hyaloplasm where it bounds *C* and *B*, and *A* only yolk. Evidently, the substances are distributed passively and arbitrarily over the blastomeres according to their incidental positions. However, some rearrangement has occurred, as the layers have been drawn out along the cleavage furrows when these were cutting through the egg.

Finally, a sample fixed 85 min. after centrifuging contains an 8-cell stage, with beginning of the 4th cleavage mitosis in some of its cells. The cells 1*a*, 1*A*, 1*b* and 1*B* contain only yolk; 1*D* mostly yolk with hyaloplasm occupying part of its inner side, 1*d* hyaloplasm with some scattered yolk granules, 1*c* and 1*C* only oil and hyaloplasm. The intracellular boundaries between substances have become rather unsharp. Again, one of the polar bodies has entered into a micromere.

Fragmentary as these observations may be, they show clearly that the substances separated by centrifuging are distributed in an arbitrary way over the blastomeres at cleavage. The first cleavage divisions may separate yolk-laden and yolk-free cells; the boundaries thus produced remain quite sharp at first. On the other hand, between the substances lying together in one blastomere the boundaries soon become less sharp and an interpenetration of the layers takes place.

It is a pity that in none of these centrifuged eggs an antipolar lobe was present at the time of fixation. It should have been interesting to study its composition in various cases. Some observations on living centrifuged eggs have shown that an antipolar lobe was formed at 1st cleavage in a normal way, even when it only contained oil. Moreover, these observations confirm the arbitrary distribution of the substances at cleavage; the oil zone may lie either in *AB* or *CD*, or in both; later, it may be found in any of the four blastomeres *A*, *B*, *C* or *D*.

c. *Vital staining.*

When cleavage stages are stained with neutral red or Nile blue hydrochloride, all blastomeres are stained with equal intensity. However, the antipolar lobes show a lighter colour than the rest of the egg; this may partly be due, however, to their greater transparency. On the contrary, with brilliant cresyl violet both the first and the second antipolar lobe show a somewhat darker staining than the blastomeres; moreover, they have a distinctly granular structure, as they contain a dense mass of dark violet granules. With Janus green cleaving eggs remain unstained like previous stages.

d. *Cytochemical reactions.*

Like previous stages, cleaving eggs yielded only negative indophenol oxidase, benzidine peroxidase and vitamin-C reactions. The reaction with sodium nitroprussiate on fresh 4- and 8-cell stages gave a weak

colouration of the nuclear areas. After treatment with trichloroacetic acid a rather strong reaction occurs, which is confined to the immediate neighbourhood of the nuclei or cleavage spindles. When an antipolar lobe is present, this remains colourless, or the reaction extends only into its basal part. The oil zone of centrifuged cleavage stages stains deeply with Sudan III and stains red with Nile blue sulphate in the reaction of L. SMITH.

4. The trochophore stage.

Descriptions of the trochophore stage of *Sabellaria* have been given by HORST (1881), WILSON (1929) and NOVIKOFF (1938a).

According to WILSON, in *Sabellaria alveolata* the embryos show the first signs of movement at 18–20 h. An embryo 27 h. after fertilization has a rather irregular outline. It is surrounded by the crumpled fertilization membrane through which cilia project. Just posterior to the equator there is a complete ring of very short fine cilia, the first sign of the prototroch. At the anterior pole a few very fine long cilia form an apical tuft. As the embryo develops its outline loses its irregularity, and, owing to a slight increase in size, it fills up the space inside the fertilization membrane, smoothing out the wrinkles. This membrane persists, forming a rather close-fitting envelope separated from the surface of the larva by a narrow space.

About 46 h. after fertilization the gut can be seen to be differentiating. The prototroch consists of a single row of fine cilia completely surrounding the body just behind the equator. At the posterior end a single extremely fine cilium, which persists for some considerable time, has appeared. There is a slight depression in the region of the future mouth. Fifteen hours later, one long and one short provisional bristle have appeared on each side. The general colour is an unevenly distributed yellowish green by transmitted light. Shortly afterwards the mouth appears as a small well-ciliated invagination situated just behind the prototroch in the ventral depression previously mentioned. About the same time the prototroch acquires a second and posterior row of very short cilia and becomes interrupted by a gap on the dorsal surface. First one and then another bristle appears in each bundle, and the yellowish green pigment, together with specks of brown, begin to be aggregated into chromatophores. The prototroch is raised up on a backwardly projecting fold which runs round the ventral surface just in front of the mouth and passes up on each side, but does not extend on to the dorsal surface. A neurotroch of short cilia appears on the ventral surface, and the forwardly directed oesophagus becomes ciliated. The apical tuft loses its longest cilia but persists as tufts of short fine cilia throughout pelagic life.

Just over five and a half days after fertilization, the larva has reached

what can be regarded as the fully developed trochophore stage. The most striking feature is the backwardly projecting fold overhanging the mouth in front and passing round each side until it is lost as it merges into the dorsal surface. The region anterior to this fold is referred to as the "hood". Behind the hood-fold there is on either side of the mouth a second rather similar fold or ridge which in later stages becomes more prominent; this structure is called the "lip-fold". The prototroch is carried near the edge of the hood-fold, it is complete ventrally but there is a large dorsal gap. It consists of an anterior row of long and rather strong driving cilia which form the chief swimming organ of the larva at this stage. Immediately behind a second row of shorter cilia occurs; behind this there is a third line of short cilia along the edge of the hood-fold and more cilia occur in the groove formed by the hood-fold, and also on the lip-folds. On the ventral surface between the mouth and the anus there is a rather deep wide groove, which is well ciliated by a broad neurotroch. The mouth leads into a ciliated oesophagus which runs forward, to open near the anterior end of the hood into a large globular stomach, also ciliated. A short ciliated intestine passes to the anus which is situated on the ventral surface close to the posterior end of the larva. On the anterior extremity of the hood, some tufts of fine apical cilia occur, while at the posterior extremity of the body a single fine cilium projects backwards. In the dorsal gap of the prototroch there is a prominent dorsal hump, and this carries a number of long fine but rather stiff cilia which occasionally vibrate. Four long provisional bristles project from a conspicuous chaeta-sac situated on each side, and they pass out below the lip-folds. The chaeta-sacs are moved by a series of muscles. The bristles are surrounded by rings of teeth, the points of which are directed towards their distal extremities; the teeth are longer on one side of the bristle than on the other. The larva is fairly transparent and has a considerable number of oily globules in its tissues. A conspicuous feature are the fairly numerous irregular chromatophores; they have a fairly sharp irregular outline and a greenish yellow ground-colour, over which are scattered many irregular dark brown specks. One red eye-spot has appeared on the left side in front of the place where the prototroch stops short. This and the eye-spots that develop later lie deeper in the tissues than do the chromatophores.

The development of the trochophore of *Sabellaria vulgaris*, as described by NOVIKOFF (1938a), agrees in all essential points with that of *Sabellaria alveolata*.

a. *Cytological observations.*

After 24 h. of development at room temperature of about 20° C, most larvae have still a rather irregular outline and swim by an irregular rotating movement. Other larvae have become more regular, broadly spindle-shaped and swim more actively. A well-developed prototroch

and apical tuft are present; yellowish green pigment cells are visible in the ectoderm.

Interpretation of the sections is rather difficult because of the compact nature of the tissues and the indefiniteness of cell limits. The ectoderm closely surrounds the central mass of endoderm; its thickness varies in different places, but it is not possible to distinguish special differentiations. In the endoderm, the future regions of the gut cannot yet be recognized; in places the formation of a lumen is indicated by the arrangement of the cells. The mouth is visible as a narrow invagination on one side of the embryo, piercing the ectoderm but scarcely penetrating into the endoderm mass. In some places, rather small cells are found between ectoderm and endoderm which are supposed to be mesoderm cells. In other places, the ectoderm and endoderm are separated by a cavity, which often seems to be lined by a delicate membrane; presumably, this is a remnant of the blastocoelic cavity. In the ectoderm, pigment cells filled with brownish granules may be seen. The prototrochal cilia are still very delicate; the apical tuft has not been found in the sections. Beneath the prototroch in many places a cavity is found; these cavities are not forming a continuous canal. In places, a nucleus is seen lying immediately against the cavity, which is probably intracellular. The polar bodies, containing pycnotic nuclei, are still present in the interior of the larva; as a rule, they are lying between ectoderm and endoderm.

On an average, the nuclei of the endoderm are bigger and clearer than those of the ectoderm. FEULGEN preparations show that the nuclei are very different in size and stainability; many resting nuclei are, however, strongly FEULGEN-positive at this stage. The pycnotic nuclei of the polar bodies are still intensely stained in these preparations. The prototrochal cells have big clear nuclei, which are only very weakly stained in FEULGEN preparations. Mitoses are still very numerous in the endoderm, whereas in the ectoderm only a few dividing cells are present.

Big spherical yolk granules are present in great number; they show a characteristic distribution. A continuous layer of these granules is present in the ectoplasm of the ectoderm cells; at the mouth, they pass inwards lining the stomodaeal invagination. The lumen of the "prototroch canal" is surrounded by a single layer of yolk granules. Furthermore, numerous yolk granules are present in the sinus-like blastocoelic cavities surrounding the endodermal mass. In the latter, only few scattered yolk granules are present. The yolk granules are about uniform in size, but they differ in their staining properties. Most granules are black after iron haematoxylin-eosin staining, orange-coloured in azan-stained sections; fewer ones stain red with eosin and blue after azan staining. The latter kind of yolk granules are found chiefly in the blastocoelic cavities and in the endoderm, only sporadically also in the ectoderm. Presumably, this change in colourability indicates a beginning digestion of the yolk. The observation that many yolk granules are found extra-

cellularly in the blastocoelic cavity, and that in these granules a high proportion shows the change in staining properties, points to a partial extracellular digestion of the yolk by proteolytic enzymes secreted into this cavity; it is not easy, however, to prove this supposition.

Twenty-nine hours after fertilization, nearly all larvae have developed to actively swimming spindle-shaped trochophores. On microscopical examination of the sections, no great changes as compared to the previous stage can be observed. The apical tuft is now distinctly visible. In the praetrochal part of the body, gland cells containing a big spherical vacuole begin to differentiate. The contents of the vacuole stain pink with eosin, blue in azan-stained sections. An ectodermal thickening on both sides of the body form the anlagen of the chaeta-sacs; the cells in this region have big oval pale nuclei.

In FEULGEN preparations, the nuclei show great differences in size and staining properties. In general, the smaller resting nuclei show a deep red-violet staining; bigger ones are less deeply stained and their colour is, in these preparations, counterstained with light green, more blue-violet. Chromosomes of dividing cells are deep red. On an average,

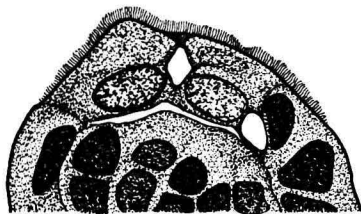


Fig. 10. *Sabellaria*, trochophore. FEULGEN. Prototroch cells with pale nuclei.

the staining of the endodermal nuclei is perhaps somewhat deeper than that of the ectodermal ones, but this difference is not always clearly visible. A few structures are characterized by nuclei which are scarcely FEULGEN-positive at all; they are 1. the prototrochal cells, with big oval nuclei which are very pale in FEULGEN preparations (fig. 10); 2. the "prototroch canal", the few nuclei of which are also extremely pale; 3. the anlagen of the chaeta-sacs, which differ from the surrounding ectoderm by their lightly-staining nuclei.

In UNNA-BRACHET preparations, all cells of the larva show an even red staining; no differences between the cells can be observed. In particular, the prototrochal cells do not differ from the rest of the larva.

After CHAMPY fixation and staining with saffranin, in the ectoderm a special kind of highly saffraninophil cells can be observed. They are triangular in cross-section, with broad base directed towards the periphery, and apex penetrating between the other ectodermal cells which are columnar in shape (fig. 11). There are some indications that they have something to do with the differentiation of pigment cells.

Larvae of 35 h. are all spindle-shaped trochophores. The 3 sections of the gut: oesophagus, stomach and intestine can be distinguished. The latter ends at the anus near the posterior end of the body (fig. 12). In the stomach and intestine a distinct lumen has appeared; in the oesophagus the lumen is indicated. In some places, especially on the dorsal and apical side of the stomach, the gut is separated from the ectoderm by a clearly defined blastocoelic sinus (fig. 13); elsewhere, some sporadic mesoderm cells are lying between ectoderm and endoderm. The anlagen of the chaeta-sacs have invaginated to well-developed globular structures, lying in the posterior part of the body on either side of the intestine. In transverse sections, they consist of 4–5 cells

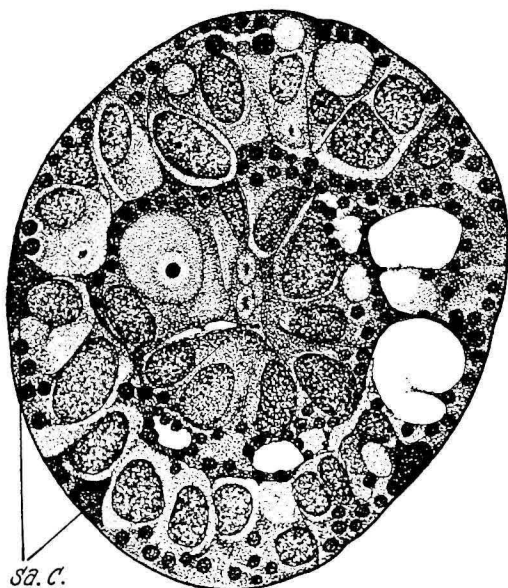


Fig. 11. *Sabellaria*, trochophore, 29 h. CHAMPY, saffranin. Saffraninophil cells (sa. c.) in ectoderm.

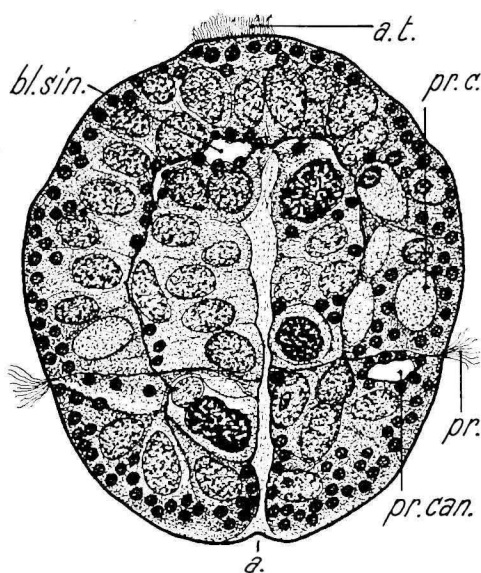


Fig. 12. *Sabellaria*, trochophore, 35 h. Longit. section of intestine and anus (a.) Prototroch (pr.) and prototrochal septum. Prototroch cells (pr. c.) with big clear nuclei. pr. can. = prototroch canal. a. t. = apical tuft. bl. sin. = blastocoelic sinus.

with big oval and very transparent nuclei, arranged around a small round central lumen. In some cases, the very first rudiments of the bristles have appeared as short dark vaguely defined rods between the cells. The gland cells in the praetrochal part of the body are well-differentiated. Apparently, there are three such cells, arranged in a triangle around the apical tuft, about half-way between this and the prototroch. One is situated medio-ventrally above the mouth; it is bigger than the paired laterodorsal ones. Each of these gland cells contains a big spherical vacuole filled with a substance staining pink with

eosin and blue with azan, probably mucus. The prototrochal cells are still characterized by big transparent nuclei. The roots of the prototrochal cilia seem to join into a plasmatic lamella, which extends inwards towards the gut, forming a kind of horizontal septum separating the praetrochal and posttrochal parts of the body (fig. 12). The "prototroch canal", lying near this lamella, still seems to consist of a number of discrete cavities not connected with one another. The polar bodies with pycnotic nuclei are still to be found beneath the ectoderm. Big spherical yolk granules are still present in great number in the ectoplasmic part of the ectoderm cells and in the blastocoelic cavity (fig. 13). The lumen of the prototroch canal is surrounded by a single layer

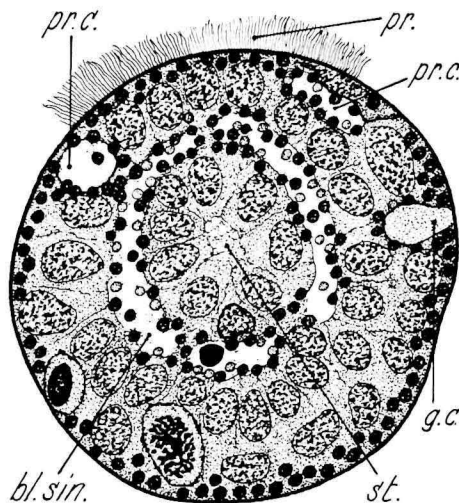


Fig. 13. *Sabellaria*, trochophore, 35 h. Transverse section. Blastocoelic sinus (bl.sin.) surrounding stomach (st.). Prototroch (pr.) and prototroch canal (pr.c.). Gland cell (g.c.).

of these granules; furthermore, sporadic granules are found in the outer part of the endoderm cells and along the lumen of the posterior part of the intestine (fig. 12). Part of the yolk granules in the blastocoelic sinus and in the endoderm exhibit the change in colouration which is supposed to indicate that they are being digested.

A sample of larvae 27 h. old, which presumably had developed at a somewhat higher temperature, are at a slightly more advanced stage of development. The bristle rudiments are somewhat longer and have reached about half the length of the chaeta-sacs, in which they are formed; they do not yet protrude externally. The number of yolk granules staining blue in azan-stained sections has greatly increased; such granules are also found in the ectoderm now. Apparently, yolk digestion is very active at this stage. As a matter of fact, the total amount of yolk has clearly diminished by now. In the ectoderm, accumulations of a dense

granular substance, staining a rusty brown in iron haematoxylin-eosin preparations, have appeared; their nature is still obscure.

Forty-eight hours after fertilization, the larvae are trochophores with 1—2 pairs of provisional bristles, the longest of which reach $\frac{1}{3}$ — $\frac{2}{3}$ of the body length of the larvae. The apical tuft is still well-developed.

The ectoderm contains a great deal of the eosinophil substance mentioned above, distributed in the form of granular masses within the cells. In the neighbourhood of the apical pole, these masses show a rather regular symmetric arrangement. The gut has a well-defined lumen and

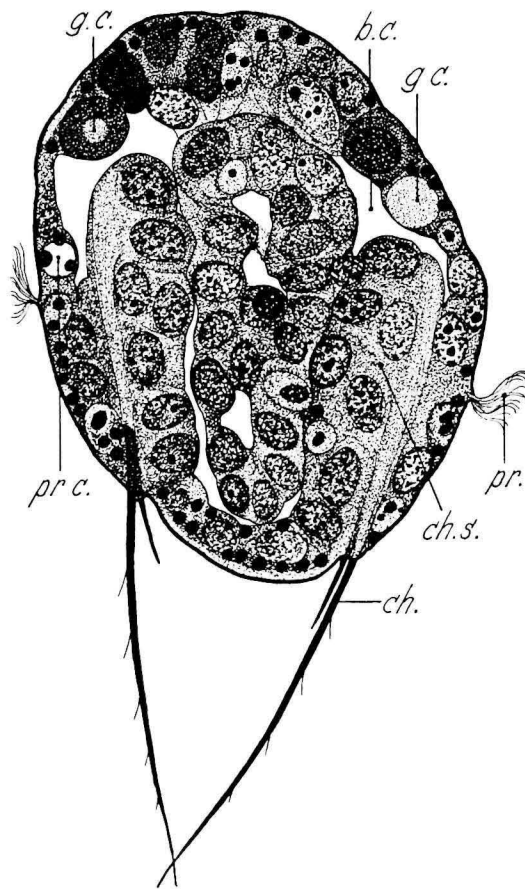


Fig. 14. *Sabellaria*, trochophore, 48 h. Longitudinal section. Chaeta-sacs (ch.s) with protruding bristles (ch.). Prototroch (pr.) and prototroch canal (pr.c.). Gland cells (g.c.) in praetrochal part. Open body cavity (b.c.).

is ciliated throughout. In many larvae, a distinct open body cavity has appeared between the anterior part of the gut and the ectoderm (fig. 14); the yolk granules in the blastocoel have disappeared. The prototroch has become interrupted by a gap on the dorsal side. The horizontal septum at this level is clearly visible. The apical tuft is well-developed;

the ectoderm at its base forms a kind of thickened cushion, which contains some clear nuclei in its central part beneath the apical tuft. The lumina of the prototroch canal in most sections are situated slightly above the level of the prototroch. The praetrochal gland cells have become pear-shaped, the secretion vacuole protruding with a conical projection towards the surface. The inner part of these cells, with a dense protoplasm, projects beneath the ectoderm into the body cavity. Chromatophores with numerous pigment granules are present in the praetrochal part of the ectoderm. The chaeta-sacs have considerably lengthened, reaching about $\frac{3}{5}$ the length of the body (fig. 14). Transverse sections show that besides the two bristles which have emerged a third is being formed in the chaeta-sacs. In many larvae the pycnotic nuclei of the polar bodies still can be observed in the blastocoelic cavity or between

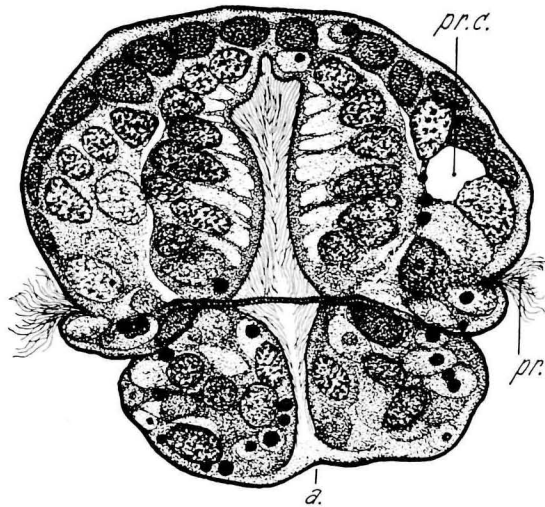


Fig. 15. *Sabellaria*, trochophore, 72 h. Longitudinal section of intestine and anus (a). Prototroch (pr.) and prototrochal septum. Prototroch canal (pr.c.) in praetrochal part of body.

the ectoderm cells. The amount of yolk has greatly diminished; in the ectoderm, yolk granules are still rather numerous in the outer part of the cells, especially in the posterior half of the body; in the inner tissues, however, the number of yolk granules has become extremely small.

Six hours later, hence 54 h. after fertilization, the longest provisional bristles have a length equalling or slightly surpassing the body length of the larva. The lumen of oesophagus, stomach and intestine has become somewhat more sharply delimited by a distinct line, blue in azan-stained sections. The hoodfold, overhanging the mouth, has appeared. The lumen of the prototroch canal is now clearly situated above the level of the prototroch. In the chaeta-sacs the rudiment of a fourth bristle is being formed.

Finally, larvae 72 h. old possess 3 pairs of provisional bristles, the

longest of which are $1\frac{1}{2}$ to 2 times the length of the body. The apical tuft has disappeared. The wall of the gut shows a peculiar structure. The nuclei are situated in the outer part of the cells, whereas the inner part consists of a very clear watery protoplasm, which appears nearly empty in the sections. Towards the lumen, this is bounded by a narrow eosinophil zone, which bears the cilia (fig. 15). At the level of the prototroch, the above-mentioned septum is well-developed. It is attached to the gut at the transition between stomach and intestine; the gut lumen sometimes shows a conspicuous dilatation at this point (fig. 15). The lumen of the prototroch canal is situated in the praetrochal part of the body, at some distance above the prototroch; it is surrounded by pigment granules. The yolk has nearly entirely been consumed; only in the ectoderm of the posttrochal part of the body and the wall of the intestine still some yolk granules can be observed.

Discussion.

1. The distribution of substances.

a. *Yolk.*

In the newly-shed eggs, the yolk occupies the rather narrow marginal layer surrounding the germinal vesicle. After the dissolution of the latter, the yolk granules spread throughout the cytoplasm, leaving free only the areas of spindles and asters and the hyaline cortical zone of the egg. Whereas in centrifuged unfertilized eggs the yolk granules, at first sedimented at the centrifugal pole, later show a clearly-marked tendency to accumulate around the maturation spindle, no such concentration of the yolk around the spindles can be observed in normal development. On the contrary, also in cleaving eggs the yolk remains quite evenly distributed throughout the cytoplasm. The contents of the first and second polar lobe do not differ in density or staining properties of the yolk from the rest of the egg. Only towards the end of the 4-cell stage the density of the yolk seems to increase slightly on the vegetative side of the blastomeres; the third polar lobe also shows a somewhat greater yolk density. In the gastrula, a peripheral layer of yolk granules beneath the egg surface and along the archenteron lumen is very conspicuous. The interior of the cells contains much less yolk; in the endoplasm for the first time a change in staining properties of some of the granules, indicating a beginning digestion of the yolk, becomes visible at this stage. In the trochophore, besides the intracellular digestion also an extracellular digestion of the yolk in the blastocoelic cavity seems to take place. In this way, a rapid decrease in the amount of yolk occurs. For some time, rather numerous yolk granules remain visible in the outer part of the ectoderm cells, especially in the posterior part of the body. In the 3-days old larvae, however, even these have nearly entirely disappeared.

b. *Fat.*

Only few observations have been made on the distribution of fat globules. In general, these globules seem to agree with the yolk granules in their distribution. Cytochemical reactions on centrifuged unfertilized eggs have shown that the fat zone of these eggs contains lipins and unsaturated glycerides. Staining of centrifuged eggs with Sudan III revealed that the yolk granules contain a certain amount of lipid substances; this seems to diminish with fertilization.

c. *Phosphatides.*

After CHAMPY fixation and staining with HEIDENHAIN's iron haematoxylin, peculiar black vesicles of different sizes are found in the cytoplasm. Presumably, they consist of phosphatides; their appearance in the sections may be partly due to the action of the fixing fluids. Whether they arise from preformed granules or from phosphatides dissolved in the cytoplasm is not certain. Both in unfertilized and fertilized eggs and during cleavage they are especially concentrated around the nuclei and spindles. Evidently, the perinuclear cytoplasm is very rich in phosphatides.

d. *Thymonucleic acid.*

In newly-shed eggs, the germinal vesicle is entirely FEULGEN-negative. However, already before the dissolution of the nuclear membrane thymonucleic acid begins to accumulate in chromatin granules. The chromosomes of the first maturation division are at first thread-like with little FEULGEN-positive thickenings at regular distances. The sperm nucleus, immediately after it has entered the egg, is only weakly FEULGEN-positive. Later, granules rich in thymonucleic acid appear on its surface. After the formation of the polar bodies, these contain deeply-stained short chromosomes which fuse to a single pycnotic body; they retain their thymonucleic acid for a long time, at least to a trochophore stage of 24 h. The pronuclei are very weakly FEULGEN-positive. At prophase of the first cleavage division, delicate chromatin threads, forming large spirals, and in which small FEULGEN-positive chromomeres are lying, become visible. They condense into the metaphase chromosomes. When at telophase the chromosomes swell into karyomeres, their FEULGEN-staining diminishes. The resting nuclei show scarcely any staining. At the next cleavage the same cycle is repeated: increase of staining during spiralization from prophase to metaphase, decrease during swelling from telophase to resting nuclei.

At the blastula stage, a distinct increase in the total thymonucleic acid content has occurred; now also the resting nuclei are clearly FEULGEN-positive, though their colourability still decreases with swelling. In later stages of development, this staining of the resting nuclei becomes still

more pronounced. A similar phenomenon, indicating an increased synthesis of thymonucleic acid towards the end of cleavage, has also been observed in sea urchin, *Barnea*, *Rana* and Axolotl (BRACHET 1933), in *Rhabditis* (STEFANELLI 1940) and in *Limnaea* (RAVEN 1946). At the gastrula stage, a beginning differentiation of the cells is indicated by great differences in thymonucleic acid content between nuclei of nearly equal size. This becomes more pronounced in the trochophore. The prototrochal cells are characterized by big clear nuclei which are poor in thymonucleic acid. The same holds true of the nuclei of the "prototroch canal" and those of the chaeta-sacs of the provisional bristles. Evidently, the cells of the larval organs, which have reached a functional phase early in development, are characterized by nuclei poor in thymonucleic acid, in contrast to other cells, which by repeated divisions build up the organism. This agrees with similar observations in *Limnaea* (RAVEN 1946).

e. *Ribonucleic acid.*

In the newly-shed eggs, besides the nucleolus the cytoplasm contains ribonucleic acid. The outer hyaline plasm zone stains rather heavily with pyronine; deeply-staining masses are also found on the outer side of the nuclear membrane. In the rest of the cytoplasm, the staining is restricted to the protoplasmic meshes between the nearly colourless yolk granules. After the dissolution of the germinal vesicle, the nuclear membrane nucleotides remain visible for some time as a dark ring. Near the entrance point of the sperm perhaps a temporary decrease in pyroninophily occurs. The sperm head is rich in ribonucleic acid. Furthermore, the maturation and cleavage spindles are deeply stained with pyronine. An accumulation of ribonucleic acid in or against the nuclear membrane of pronuclei and cleavage nuclei is clearly visible. The polar bodies are rich in ribonucleic acid.

At the blastula stage, the ectoplasm is somewhat more pyroninophil than the endoplasm. Cells in mitosis have a less basophil cytoplasm than resting cells. The nuclei contain ribonucleic acid in the nucleoli and nuclear membranes.

In the trochophore, no differences in ribonucleic acid content between the cells can be observed.

f. *Sulphydril compounds.*

In freshly-shed eggs, the nucleoplasm of the germinal vesicle contains free glutathione in its reduced form. After the dissolution of the germinal vesicle, this substance spreads through the cytoplasm, but remains most concentrated around the spindles and nuclei. Bound sulphydril compounds are present in the whole cytoplasm, but especially in its central part; during cleavage, they are restricted to the immediate neighbourhood of the nuclei and cleavage spindles.

The presence of free glutathione in the nucleoplasm of the germinal vesicle and around the nuclei of later stages seems to be of a fairly general occurrence (BRACHET 1940, RAVEN 1946).

2. The vital staining experiments.

The vital staining experiments have failed to show anything resembling a "bipolar differentiation" in the eggs of *Sabellaria*. As a matter of fact, in centrifuged eggs stained with neutral red the yolk zone becomes deep-red, whereas the hyaloplasm gets an orange-brown colour. This differential staining of yolk and hyaloplasm in an "acid" and "alkaline" tint, respectively, corresponds to the observations in other eggs (RAVEN 1938). Since the yolk in the *Sabellaria* egg scarcely accumulates at one end in normal development, these differences in staining property do not lead, however, to a "bipolar differentiation" in this egg. This corroborates the conclusion drawn in the above-mentioned paper that the "bipolar differentiation" in those eggs where it is observed, is due to shifting of the yolk. Only the slight increase in yolk density at the vegetative end of the cells towards the end of the 4-cell stage is a comparable phenomenon, but it is too small in extent to lead to clear differences in staining between the poles.

3. The antipolar lobes.

The antipolar lobes of the *Sabellaria* egg which appear 3 times, during first, second and third cleavage, respectively, do not show any clear differences in composition compared with the rest of the egg. In normal development, they consist of cytoplasm containing many yolk granules. Yolk density in the polar lobes does not differ from that in the remaining part of the egg; only in the 3rd lobe it is somewhat greater, in consequence of the slight concentration of yolk at the vegetative end which precedes its formation. After centrifuging, the polar lobes may contain hyaloplasm or oil only; still, they are formed in a normal way. This agrees with the observations made in eggs of *Ilyanassa* (MORGAN 1935) and *Chaetopterus* (RAVEN 1938) proving that the formation of the lobe is rather independent of the nature of the substance it contains.

In connexion with the importance of the lobes for morphogenesis, and the fact that they contain the determining factors of the apical tuft and posttrochal region of the larva (HATT 1932, NOVIKOFF 1938b), their cytochemical composition has been studied with great care, in order to determine if any differences with the rest of the egg could be observed. The results of this study are mainly negative; no clear differences have been observed. In particular, it must be emphasized that no special accumulation of ribonucleic acid or sulphhydryl compounds in the region of the lobes occurs, and that the oxidative enzymes studied

cannot be demonstrated in the *Sabellaria* egg at all. Also, the peripheral hyaline zone of the egg which has become rather narrow at these stages, is not markedly thickened in the region of the lobes. The only observation which might be explained as pointing to a different physico-chemical composition of the lobes is their somewhat greater stainability with brilliant cresyl violet and the occurrence of dark granules with this staining. The interpretation of this fact remains difficult, however.

It must be concluded, therefore, that our present cytochemical methods, as far as they have been used by us, are unable to demonstrate in all cases the "chemodifferentiation" which is to be expected on the basis of our knowledge of the morphogenetic properties of the parts of the germ. This may be due to the still unsatisfactory state of our present possibilities of cytochemical analysis, especially as regards the proteins. It may be assumed that the special properties of "morphogenetic plasms" in most cases will be due to the structural peculiarities of their protein components, which up till now are quite beyond the reach of our cytochemical analysis. On the other hand, the possibility must be left open that these special morphogenetic properties might not be due to a different chemical composition of these plasms, but rather to physical factors of a dynamic nature which, most probably, will be located in the cortical layer of the eggs.

In this connexion it is of some interest to consider the possibility that the special morphogenetic properties of the polar lobes do not reside in their internal cytoplasm, but are rather bound to their cortical layer. The observation that polar lobes are formed in centrifuged eggs even when their contents are quite abnormal, points to the dynamic part played by the cortex in their formation and form changes. The possibility cannot be denied that the latter is also responsible for their morphogenetic role in further development. A relatively simple experiment presents itself to the mind which might answer this question: provided that centrifuged eggs of forms with clear polar lobes develop to normal larvae, like those of many of their relatives, what influence will have the removal of a polar lobe containing e.g. only oil? If it corresponds to the removal of a normal lobe, it may be concluded that, indeed, cortical factors are responsible for the special morphogenetic properties of the lobes; if it does not, the internal cytoplasm of the lobe does also play a part. Centrifuge experiments with the eggs of *Ilyanassa* have been made by MORGAN (1935), but they give no clue with respect to our question. We hope to continue our experiments with *Sabellaria* in this direction in the near future.

4. The polar bodies.

In the course of our observations, a peculiar behaviour of the polar bodies in *Sabellaria* has been noted. At the 8—12 cell stage, these bodies

are incorporated into some of the micromeres at the animal pole. At the blastula and gastrula stages they are found between the cells in the interior of the embryo, near the cleavage cavity. In the trochophore stage, they are mostly lying in the cleavage cavity; here they may be observed even in a 2 days old embryo. Their ultimate fate could not be determined; it may be that they finally disintegrate, but no signs of it have been observed. Their pycnotic nuclei remain sharply delimited till the end, and even in a trochophore are still very rich in thymonucleic acid.

Similar observations have been made in other forms. GROBBEN (1881) found in *Cetochilus* that at least one polar body wanders into the segmentation cavity, and a similar fate for the polar globules has been described by HATSCHEK (1885) in *Eupomatus* (cited after TREADWELL 1901). According to MEAD (1897), in *Lepidonotus* at the 32-cell stage the polar globules penetrate into the cross cells; they may enter the same, adjacent, or even opposite cells, and rarely one works its way between the cells into the segmentation cavity. In *Chaetopterus*, the polar bodies similarly are ingested by the rosette cells. EISIG (1898) reports that the polar bodies of *Capitella* remain for 1—2 days at the upper pole of the egg, whereafter they sink, showing signs of degeneration, into the egg and finally disappear. According to TREADWELL (1901), in *Podarke* at the 32-cell stage the polar bodies pass into the ectomeres, where they may be seen as small, deeply staining bodies, lying in the protoplasm of the cell. This position they retain for some time. Later, dark bodies are seen lying inside the segmentation cavity, and later still they touch the endoderm. The author doubts, however, their identity with the polar bodies and suggests that they are derived from the ectoderm.

It appears from these observations that the ingestion of the polar bodies by the animal micromeres and their migration toward the cleavage cavity is of rather common occurrence in Annelids. The ultimate fate of these bodies, and the possible significance of the process for later development, remain obscure up till now. It might be of some interest to devote attention to this point in future investigations on Annelid development.

5. The Sabellaria trochophore.

The trochophore stage of *Sabellaria* exhibits some structures which deserve special attention, as they have not been described in this species up till now.

a. *The prototroch canal.* Already in the earliest trochophore stage a row of cavities is present beneath the prototroch cells. Although they are not mutually connected, their linear arrangement suggests that

they form part of a common structure underlying the prototroch and which will be called the prototroch canal. The cavities are probably intracellular; nuclei are seen in places lying immediately against the cavity. Furthermore, the cavities are surrounded by a single layer of big yolk granules. The nuclei are pale and poor in thymonucleic acid.

In later stages, the structure shows a gradual shift with respect to the position of the prototroch; it moves more and more upwards, so that in the oldest stage studied (72 h.) it is clearly situated in the praetrochal part of the body, at some distance above the prototroch. Still, the lumina are disconnected; whether they will coalesce to a common duct at later stages is not certain.

Probably, this structure corresponds to what has been provisionally described as the "head kidney" by WILSON (1892) in the *Nereis* trochophore. Here, it arises from a pair of cells (the cephalic nephroblasts), which are derived from the posterior micromeres (c_1 , d_1), and soon sink below the surface; then, they slowly migrate outwards and downwards towards the prototroch, forcing their way between the outer layer of cells and the four endomeres. Meanwhile they become more elongated and somewhat pointed at the ends. They pass downwards until they lie quite in the lower hemisphere below the prototroch, where the remainder of their development is accomplished.

Each nephroblast rapidly elongates, extending itself forwards and backwards between the outer cells and the endomeres. It is thus converted into an elongated organ which extends about half-way around the body.

At an early stage, the protoplasm becomes clear and vacuolated; the vacuoles always appear at one side of the cell, the nucleus being crowded to one side. As the nephroblast elongates, the vacuoles coalesce so as to form a sinuous canal in the protoplasm. As the elongation proceeds, the canal becomes narrower and more distinct, and the head-kidney is thus converted into a slender tube.

When fully formed, the head-kidneys entirely surround the body and again lie partly inside the prototroch. In the latest stages in which it was observed, the head-kidney lies completely inside the prototroch, considerably flattened against the cells of the latter. Its ultimate fate could not be determined.

The author explains that he termed this organ the head-kidney only with a certain reservation, since he did not succeed in observing any evidence of cilia in the cavity, or any sign of an external opening.

In *Amphitrite* the corresponding cells, except for a small area left at the surface, are covered over by the surrounding cells, and become huge mucous glands in the praetrochal part of the body (MEAD 1897). Later, WILSON (1896) suggests that possibly they are slime glands in *Nereis* also.

We have observed this organ in sections of *Nereis* trochophores. Its

position here corresponds entirely to that of the "prototroch canal" in *Sabellaria*. In *Nereis*, its lumen is filled with big droplets of a probably mucous substance, staining deep-blue with azan; this supports the view that it is a mucous gland. In *Sabellaria*, the cavities are always empty in the stages observed by us; hence, nothing can be said about its function in this form.

b. *Gland cells*. In *Sabellaria*, three big gland cells are formed in the upper hemisphere. They are arranged in a triangle around the apical tuft, about half-way between this and the prototroch. One is situated medioventrally above the mouth; it is the biggest of the three. The other two are symmetrically placed on the laterodorsal side. At first, they contain a big spherical vacuole, the contents of which stain pink with eosin, blue with azan. Later, they become pear-shaped, the secretion vacuole protruding with a conical projection towards the surface. The inner part of the cells, consisting of dense protoplasm, projects beneath the ectoderm into the body cavity.

These gland cells are comparable to similar structures observed in other Annelids.

In the *Nereis* trochophore, WILSON (1892) observed five spherical bodies on the anterior half of the upper hemisphere, arranged in a regular arc, one of them lying in the median line, the others symmetrically placed on either side of it. These "frontal bodies" appear to be developed each out of a single cell, in which appears a clear space like a vacuole surrounded by a layer of granular protoplasm. The clear space stains intensely with haematoxylin. Therefore, the author regards them as glands. They are at first spherical, but afterwards assume a pear-shape, the clear space extending out into the narrower portion, which is perhaps to be regarded as a kind of duct. At first separated from each other, they are later brought into contact, crowded closely together, and, after diminishing in size and becoming distorted in form, they disappear in the surrounding ectoblast.

We have studied these bodies in sections of *Nereis* trochophores, and can state that they agree entirely in general disposition, shape and staining properties to the gland cells observed by us in *Sabellaria*.

MEAD (1897) observed similar cells in *Amphitrite*, as five spherical bodies, disposed symmetrically, one on the midventral line, two on the ventral, and two on the dorsal side. They appear to be spherical vesicles filled with a fluid, which does not react to methyl-green nor DELAFIELD's haematoxylin, but does stain with ZOCHE's alum-cochineal. The author calls them "problematical bodies" and suggests that they are homologous with the frontal bodies of *Nereis*.

Similar bodies have been found in *Podarke* by TREADWELL (1901). There are two on a side, closely crowded together just in front of the eye-spot, and a fifth very small one on the median line in front. They

stain very deeply with haematoxylin, an outer portion (WILSON's "duct") staining much more deeply than the inner. From the number of nuclei surrounding each body in the early stages, the author thinks they are formed from more than one cell. Their staining reactions indicate that they have a glandular function. Probably, they have an excretory rather than a slime secreting function, the necessity for the one and not for the other being apparent.

c. *The prototrochal septum.* This structure has first been observed in a trochophore of 35 h., as a plasmatic lamella, reaching inwards from the roots of the prototrochal cilia towards the gut, at which it seems to be attached. No references to a similar structure in other Annelids have been found in the literature. Possibly, it represents the prototrochal muscle ring described in other Annelids, which accompanies the prototrochal nerve ring. The latter has not been observed in *Sabellaria*. The observation that sometimes a dilatation of the gut lumen is found at the place of attachment of the septum points to its muscular nature.

Summary.

1. The development of *Sabellaria alveolata* L. has been studied by cytological and cytochemical methods.
2. The yolk is evenly distributed through the cells until the gastrula stage. From this stage on, digestion of the yolk begins, which leads to its disappearance in the 3-day old trochophore.
3. The fat globules agree with the yolk granules in their distribution. The fat zone of centrifuged eggs contains lipins and unsaturated glycerides.
4. The cytoplasm surrounding the nuclei and spindles of maturation and cleavage stages is very rich in phosphatides.
5. From the blastula stage on, a distinct increase of thymonucleic acid synthesis takes place. At the gastrula stage, beginning cell differentiation is accompanied by differences in thymonucleic acid content of the nuclei. In the trochophore, the nuclei of the functioning larval organs: prototroch, prototrochal canal and chaeta-sacs of the provisional bristles, are poor in thymonucleic acid.
6. The distribution of ribonucleic acid exhibits no peculiarities which could be related to the determination of the cells.
7. Glutathione and bound sulphydril compounds are concentrated around the nuclei and spindles.
8. The egg of *Sabellaria* shows no "bipolar differentiation" in the sense of SPEK.
9. Except a slightly greater stainability with brilliant cresyl violet, the polar lobes show no differences with the rest of the egg pointing to a different physicochemical composition.
10. In centrifuged eggs, the substances show an arbitrary localization with respect to the egg axis. The antipolar lobe is formed in a normal way even when its contents are quite abnormal.
11. The polar bodies are ingested by the animal blastomeres at the 8—12 cell stage; later, they are found in the interior of the embryo between the cells or in the cleavage cavity. They remain visible for at least 2 days.
12. In the trochophore, the following structures are described:
 - a) the prototrochal canal, underlying the prototroch at early stages and later shifting to a somewhat higher level;
 - b) 3 gland cells in the praetrochal part of the body, presumably having an excretory function;
 - c) a horizontal septum at the level of the prototroch, which may represent the prototrochal muscle ring.

LITERATURE

- BRACHET, J., Recherches sur la synthèse de l'acide thymonucléique pendant le développement de l'oeuf d'oursin. Arch. Biol. 44, 519 (1933).
- , Etude histochimique des protéines au cours du développement embryonnaire des poissons, des amphibiens et des oiseaux. Arch. Biol. 51, 167 (1940).
- , La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'amphibiens en voie de développement. Arch. Biol. 53, 207 (1942).
- DALCQ, A. M et A. SEATON-JONES, La répartition des éléments basophiles dans l'oeuf du rat et du lapin et son intérêt pour la morphogénèse. Bull. Acad. r. Belg., Cl. Sci. (5) 35, 500 (1949).
- EISIG, H., Zur Entwicklungsgeschichte der Capitelliden. Mitt. Zool. Stat. Neapel 13, 1 (1898).
- FAURÉ-FREMIET, E., L'oeuf de *Sabellaria alveolata* L. Arch. Anat. Micr. 20, 211 (1924).
- GERSCH, M., Die Erforschung der Sonderungsprozesse während der frühen Embryonalentwicklung mit Hilfe der vitalen Färbung. Arch. exp. Zellf. 22, 548 (1939).
- GERSCH, M. und E. RIES, Vergleichende Vitalfärbungsstudien: Sonderungsprozesse und Differenzierungsperioden bei Eizellen und Entwicklungsstadien in verschiedenen Tiergruppen. Roux' Archiv 136, 169 (1937).
- HARRIS, J. A., Studies on living protoplasm. I. Streaming movements in the protoplasm of the egg of *Sabellaria alveolata* (L.). J. exp. Biol. 12, 65 (1935).
- HATT, P., La fusion expérimentale d'oeufs de *Sabellaria alveolata* L. et leur développement. Arch. Biol. 42, 303 (1931).
- , Essais expérimentaux sur les localisations germinales dans l'oeuf d'un Annelide (*Sabellaria alveolata* L.). Arch. Anat. micr. 28, 84 (1932).
- HORST, R., Over bevruchting en ontwikkeling van *Hermella alveolata* Miln. Edw. Versl. Meded. Kon. Akad. v. Wetensch., Afd. Natuurk. (II) 16 (1881).
- LEHMANN, F. E., Die Indophenolreaktion der Polplasmen von *Tubifex*. Die Naturwiss. 29, 101 (1941).
- , Zur Entwicklungsphysiologie der Polplasmen des Eies von *Tubifex*. Rev. Suisse Zool. 55, 1 (1948).
- LISON, L., Histochimie animale. Paris (1936).
- MEAD, A. D., The early development of marine Annelids. J. Morph. 13, 227 (1897).
- MORGAN, T. H., Centrifuging the eggs of *Ilyanassa* in reverse. Biol. Bull. 68, 268 (1935).
- NOVIKOFF, A. B., Embryonic determination in the Annelid, *Sabellaria vulgaris*. I. The differentiation of ectoderm and endoderm when separated through induced exogastrulation. Biol. Bull. 74, 198 (1938a).
- , Embryonic determination in the Annelid, *Sabellaria vulgaris*. II. Transplantation of polar lobes and blastomeres as a test of their inducing capacities. Biol. Bull. 74, 211 (1938b).
- , Surface changes in unfertilized and fertilized eggs of *Sabellaria vulgaris*. J. exp. Zool. 82, 217 (1939).
- , Morphogenetic substances or organizers in Annelid development. J. exp. Zool. 85, 127 (1940).
- PALADE, G. E. and A. CLAUDE, The nature of the Golgi apparatus. II. Identification of the Golgi apparatus with a complex of myelin figures. J. Morph. 85, (1949).
- PASTEELS, J., Recherches sur le cycle germinal chez l'*Ascaris*. Etude cytochimique des acides nucléiques dans l'oogénèse, la spermatogénèse et le déve-

- loppement chez *Parascaris equorum* Goerze. Arch. Biol. 59, 405 (1948).
- PELTRERA, A., Le capacità regolative dell'uovo di *Aplysia limacina* L. studiate con la centrifugazione e con le reazioni vitali. Pubbl. Staz. Zool. Napoli 18, 20 (1940).
- PITOTTI, M., La distribuzione delle ossidasi e perossidasi nelle uova di *Myzostoma*, *Beroe* e *Nereis*. Pubbl. Staz. Zool. Napoli 21, 93 (1947).
- RAVEN, CHR. P., Experimentelle Untersuchungen über die "bipolare Differenzierung" des Polychaeten- und Molluskeneies. Acta Neerl. Morphol. 1, 337 (1938).
- , The development of the egg of *Limnaea stagnalis* L. from oviposition till first cleavage. Arch. Néerl. Zool. 7, 91 (1945).
- , The development of the egg of *Limnaea stagnalis* L. from the first cleavage till the trochophore stage, with special reference to its "chemical embryology". Arch. Néerl. Zool. 7, 353 (1946).
- REVERBERI, G. e M. PITOTTI, Differenziazione fisiologiche nell'uovo delle Ascidie. Comment. Pontif. Acad. Sci. 3, 469 (1939).
- , Ricerche sulla distribuzione delle ossidasi e perossidasi lungo il "cell-lineage" di uova a mosaico. Pubbl. Staz. Zool. Napoli 18, 250 (1940).
- RIES, E., Die Verteilung von Vitamin C, Glutathion, Benzidin-peroxydase, Phenolase (Indophenolblau-oxydase) und Leukomethylenblauoxydoredukase während der frühen Embryonalentwicklung verschiedener wirbelloser Tiere. Pubbl. Staz. Zool. Napoli 16, 363 (1937).
- , Histochemische Untersuchungen über frühembryonale Sonderungsprozesse in zentrifugierten Eiern von *Aplysia*. Biodynamica 40, 1 (1938).
- , Versuche über die Bedeutung des Substanzmosaiks für die embryonale Gewebedifferenzierung bei Ascidien. Arch. exp. Zellf. 23, 95 (1939).
- RIES, E. und M. GERSCH, Die Zelldifferenzierung und Zellspezialisierung während der Embryonalentwicklung von *Aplysia limacina* L. Zugleich ein Beitrag zu Problemen der vitalen Färbung. Pubbl. Staz. Zool. Napoli 15, 223 (1936).
- SPEK, J., Zustandsänderungen der Plasmakolloide bei Befruchtung und Entwicklung des *Nereis*-eies. Protoplasma 9, 370 (1930).
- , Die bipolare Differenzierung des Protoplasmas des Teleosteer-eies und ihre Entstehung. Protoplasma 18, 497 (1933).
- , Die bipolare Differenzierung des Cephalopoden- und des Prosobranchiereies. Roux' Archiv 131, 362 (1934a).
- , Ueber die bipolare Differenzierung der Eizellen von *Nereis limbata* und *Chaetopterus pergamentaceus*. Protoplasma 21, 394 (1934b).
- STEFANELLI, A., Il rapporto nucleo-plasmatico e la sintesi dell'acido timonucleinico nello sviluppo. Ricerche sui Nematodi (*Rhabditis pellio*, BÜTSCHLI). Arch. Zool. ital. 28, 387 (1940).
- TREADWELL, A. L., The cytogeny of *Podarke obscura* Verrill. J. Morph. 17, 399 (1901).
- WATERMAN, A. J., Observations on reproduction, prematuration, and fertilization in *Sabellaria vulgaris*. Biol. Bull. 67, 97 (1934).
- , The membranes and germinal vesicle of the egg of *Sabellaria vulgaris*. Biol. Bull. 71, 46 (1936).
- WILSON, D. P., The larvae of the British Sabellarians. J. Mar. Biol. Assoc. 16, 221 (1929).
- WILSON, E. B., The cell-lineage of *Nereis*. A contribution to the cytogeny of the Annelid body. J. Morph. 6, 361 (1892).
- , Experimental studies in germinal localization. I. The germ regions in the egg of *Dentalium*. II. Experiments on the cleavage mosaic in *Patella* and *Dentalium*. J. exp. Zool. 1, 1 (1904).