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Metabolism of oocyte construction and the generation of histospecificity in the cleaving egg. Lessons from nereid annelids

ALBRECHT FISCHER*, ADRIAAN W.C. DORRESTEIJN and ULRICH HOEGER

Institut für Zoologie, Universität Mainz, Mainz, Germany

ABSTRACT The growing oocyte and the developing egg of nereid polychaetes are easily accessible to observation and experimental work, a precondition for our research. In preparation for a single semelparous act of reproduction, nereid females reutilize somatic biomass for the synchronized production of numerous oocytes. To keep oogenesis going somatic resources become recycled by the eleocytes and are supplied to the oocytes in form of vitellogenin and nucleotides (among other identified and yet unidentified substances). Both oocytes and eleocytes are free-floating coelomic cells. We postulate that availability of metabolites produced by the eleocytes might suffice to drive synchronous oocyte growth. The cortex of the fully differentiated oocyte contains numerous cortical granules which after fertilization empty by exocytosis thus causing a profound structural reorganization of the zygote cortex. Early development of nereids is extremely constant in time and spatial pattern and from the onset cleavages create diversity among the blastomeres. We have documented a correlation between the quality and amount of cytoplasm, the cell cycle duration and the histogenetic fate of such blastomeres. Experimental change of cytoplasmic proportions of early cleavage cells has serious consequences for axial development. Using a number of differentiation markers we were able to analyze the necessity of certain cleavage steps for the acquisition of the determined state.

KEY WORDS: *polychaete, oogenesis, coelomocytes, cell lineage, determination*

Introduction

Among the polychaetous annelids, a number of species transform into fast-swimming "epitokes" when becoming sexually mature. Most of them gather in the dark for spawning in coastal waters. Swarming epitokes, darting silently at the sea surface in the light of a lamp or even emitting bright green light, like the "fireworm" *Odontosyllis enopla* (Fischer and Fischer, 1995), are a fascinating view. A number of interesting biological phenomena are culminating in the swarming event: The epitokes have, by transformation, acquired a new type of musculature, developed new sets of bristles suited for swimming, are equipped with enlarged eyes and accessory sets of sensory cilia. For many of them, as for *Platynereis dumerilii* and *Nereis virens*, spawning spells the end of life because of exhaustion (a life cycle trait which has been called "catastrophic metamorphosis"; Daly, 1975). In other species only the posterior part of the segmented trunk develops into an epitoke, an independent individual (stolo) with new locomotory and sensory structures. The stolo detaches from the anterior part of the worm (stock) and carries all the mature gametes to the site of swarming (palolo worm *Eunice viridis*, many syllids as *Autolytus prolifer*). Swarming in many cases is bound to special phases of the lunar (and tidal) and diurnal cycles *(Platynereis dumerilii, Odontosyllis enopla, Eunice viridis)*.

Swarming in the open water as an unprotected epitoke, however, also means running a high risk. Therefore, most of the epitokous species invest all of their reproductive potential into this single spawning event and become moribund after swarming: All the gametes are shed at that occasion in a few seconds, and a batch of perfectly synchronized eggs is generated. Moreover, synchrony is maintained during embryonic development, and not only the time schedule of cleavages, but also their geometrical pattern is strictly invariant for a given species (Fig. 10). These have been fascinating conditions for descriptions of embryogenesis by classic embryologists (e.g. E.B. Wilson, 1892), conditions inviting as well for the experimental biologist.

We have resumed the embryological work on this favorable material using a species which has been brought into permanent culture (*Platynereis dumerilii*) by Hauenschild (Hauenschild and Fischer, 1969). On the other hand, we are studying in *Nereis virens* collected from the field the metabolism

Abbreviations used in this paper: AChE, acetylcholinesterase; PCV, packed cell volume.

***Address for reprints:** Institut für Zoologie (Abt. 1). Universität Mainz, D-55099 Mainz, Germany. FAX: 6131-393835. e-mail: AFISCHER@mzdmza.zdv.uni-mainz.de

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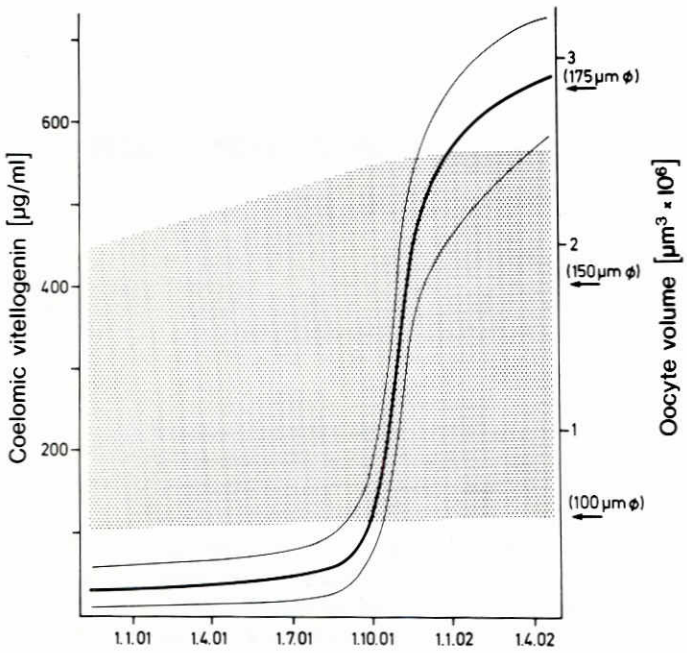


Fig. 1. Growth curve of the oocytes in a *Nereis virens* population from Yerseke/Netherlands and range of vitellogenin titers in the coelomic fluid (shaded area) of *Nereis virens* females from the same population. *Bold line: mean values of the oocyte volumes determined for a total of 317 females at different stages of oocyte development. Fine lines: standard deviation. Oocyte samples from several years were superimposed on a fictional 18 month period, beginning with November "00" (appearance of first germ cells in the coelomic fluid) and ending in April "02" when eggs are spawned in the Yerseke population. After Fischer and Hoeger (1993).*

of oocyte construction, the nature of the metabolic links between growing oocytes and maturing soma and the possible mechanisms driving and regulating these developmental processes (Fischer and Hoeger, 1993). These may appear quite divergent research topics, but there is a common dominant motif: interest in those cell-physiological mechanisms which are driving oocyte construction and in those which stand for developmental potential.

Metabolism and metabolic regulation of oocyte construction

Maturing and swarming of epitokes may be strictly scheduled by astronomic parameters as in the Palolo worm. At the same time, somatic transformation into an epitoke and the process of oogenesis both end up in perfect synchrony, and in spite of a long tradition of research on sexual maturation in nereids these processes have never been observed to dissociate under whatever conditions. Thus, epitokous transformation and oocyte growth in these animals appear linked by necessity and are offering an intriguing (and hopefully solvable) problem of regulatory physiology.

We are studying oogenesis in *Nereis virens*, a large polychaete attaining 30 g and more of body weight and thus providing ample material for biochemical analysis. Oogenesis takes about one and a half years and is well-synchronized in the population, as shown for a population in the Dutch Delta area. Oocyte growth, expressed as increase in volume, takes a modulated course, with an initial phase of extremely slow growth, a phase of rapid growth starting in September and a final phase of egg differentiation with little growth, starting in January and terminating with the spawning event in early April (Fig. 1). Lipid (in droplets), yolk protein (in yolk bodies) and mucopolysaccharides (in cortical granules) (Figs. 2,6,7) accumulate steadily with increasing oocyte size. Other compounds are getting depleted in the course of rapid growth: glycogen decreases from about 65 µmol glucose equivalents/ml PCV (packed cell volume) to about 5 µmol/ml PCV in January and finally falls off to almost nil in mature oocytes. The concentrations of adenylates, and especially of ATP, are also lowered during rapid growth. The adenylate energy charge, expressing the ratio between ATP and ADP plus AMP, already low in September (0.5), falls to some 0.15 in November, before total adenylate concentration and adenylate

Fig. 2. A full-grown oocyte of *Platynereis dumerilii*, freely floating in the coelomic cavity. *M, cross-sectioned dorsal longitudinal muscle. Semithin section, Richardson staining. Bar, 20 µm.*

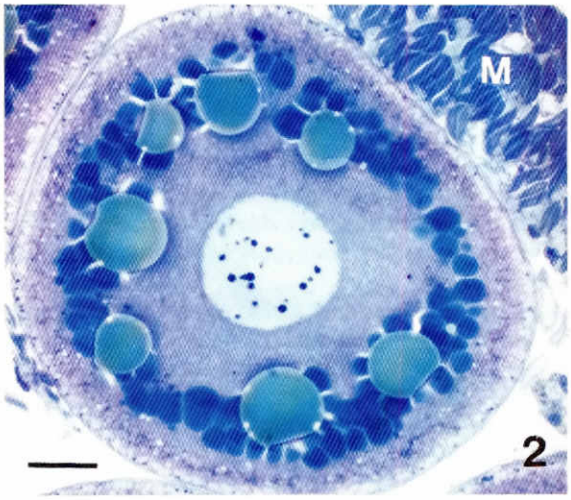
Fig. 3. Coelomic cells can easily be isolated in sufficient quantities from a single female *Nereis virens* *(see Hoeger, 1991). After washing and resuspension of the coelomic contents in Ca^{2+}/Mg^{2+}-free seawater, centrifugation at low speed (150g) yields two layers of coelomic cell types (vial at left), eleocytes (yellow) and oocytes (green layer). Sieving the resuspended pellet through nylon nets of appropriate mesh sizes (40-150 µm) separates the coelomic cells into an oocyte (middle) and an eleocyte (right) fraction.*

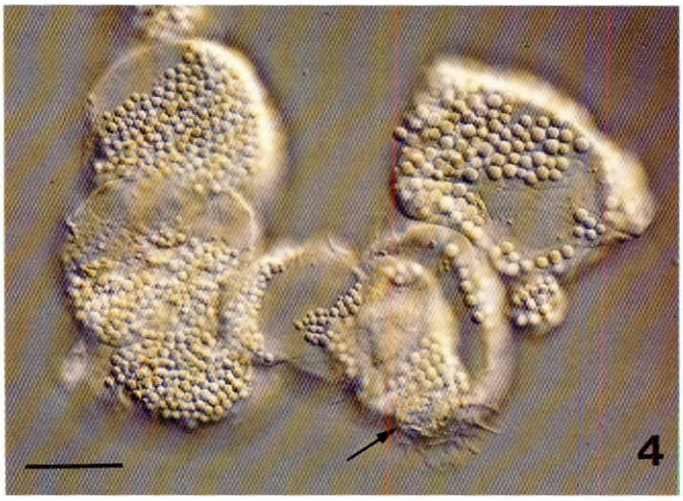
Fig. 4. A cluster of eleocytes with numerous cytoplasmic lipid droplets and a single large vacuole in each cell. *Disk-shaped granulocytes with a round nucleus (arrow) and forming pleats in the periphery are attached to the eleocytes. Nereis virens. Differential-interference-contrast. Bar, 20 µm.*

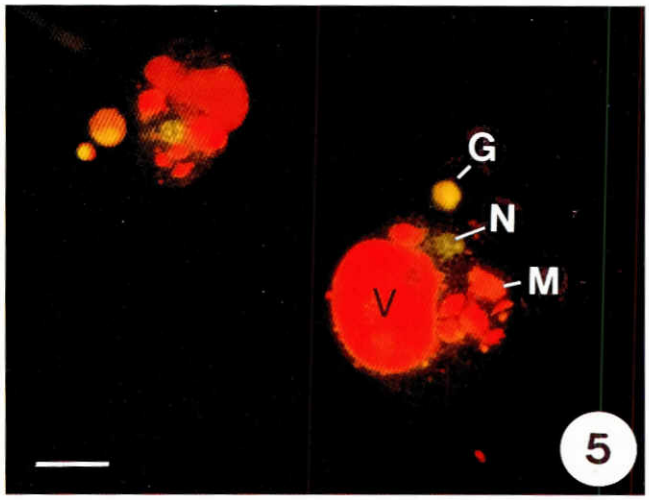
Fig. 5. Eleocytes after staining with the pH-sensitive fluorescent dye acridine orange. *The deep-orange color of the large vacuole (V) and phagocytosed muscle fragments (M) indicate the acidic milieu of these compartments, the greenish color of the surrounding cytoplasm a neutral pH. The cells were slightly compressed for better visualization of intracellular components. N, nucleus; G, attached granulocyte; epifluorescence micrograph; Nereis virens. Bar, 20 µm.*

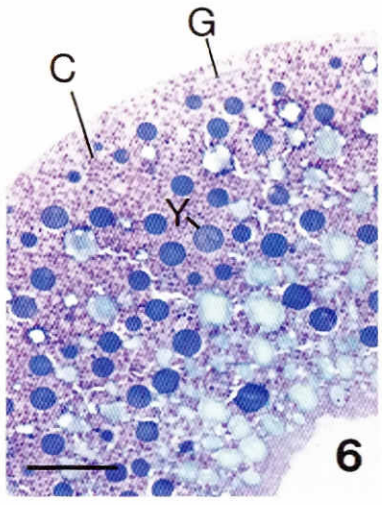
Figs. 6 and 7. Full-sized nereid oocytes undergo a rapid sorting out of cell organelles and cytoplasmic inclusions when approaching the date of spawning. *Golgi stacks (G) are displaced from the periphery deep into the cytoplasm where they disappear. Cortical granules (C) distributed all over the cytoplasm* **(Fig. 6)** *move peripherally and gather to form a multi-layered cortex* **(Fig. 7)**. *Lipid droplets coalesce into large drops of lipid, and these together with the yolk bodies (Y) form a shell between the cortex and the perinuclear cytoplasm. The whole process takes some days in the eggs of Nereis virens (shown here), but only 36 h in Platynereis dumerilii. Semi-thin section; Richardson staining. Bar, 20 µm*

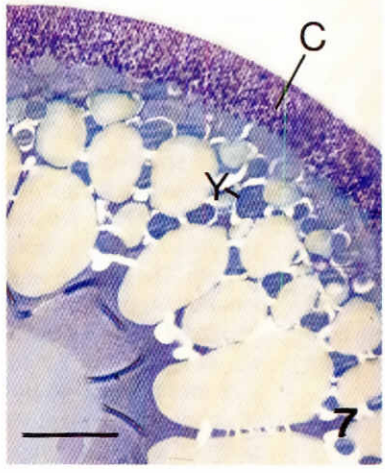
Fig. 8. Late anaphase of the first cleavage in *Platynereis dumerilii* (DIC-microscopy). *This extremely unequal cleavage allocates the ooplasmic constituents to a smaller (AB) and a much larger (CD) blastomere. The major part of the yolk-free cytoplasm is shunted into the larger CD-blastomere disproportionately to the relative volume of this blastomere. Karyomeres (arrowheads), i.e., chromosomes individually surrounded by nuclear envelope anlagen, are visible within the yolk-free cytoplasm of the CD-cell. Bar, 20 µm. (Micrograph courtesy of Dr. B. Kluge).*

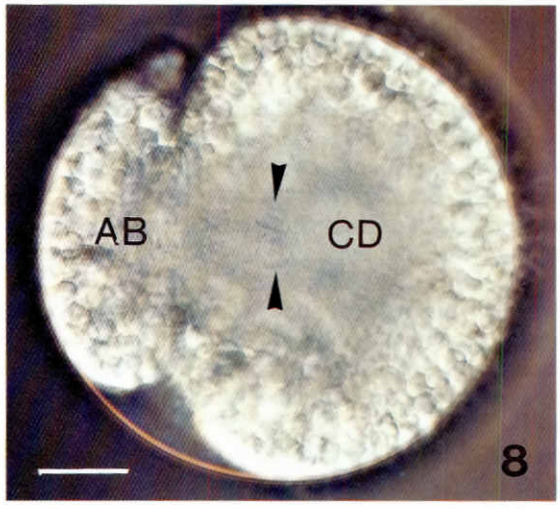












energy charge recover to 1-2 µmol/ml PCV and about 0.75, respectively, in spring (Rebscher and Hoeger 1995). These facts attest a high demand of energy-rich metabolites during rapid growth and might indicate a high level of anabolism in the oocyte. In support of this view, addition of yolk protein to the medium lowered the ATP/ADP ratio in cultured oocytes indicating that a significant part of the overall energy metabolism is recruited for the processes associated with vitellogenin uptake and/or further processing (Rebscher and Hoeger, unpublished results).

Since we are mainly interested in the control of oocyte growth, we have set out to determine the metabolite concentra-

tions available and their possible control function for oocyte growth.

We have found the conditions in *Nereis virens* perfectly suited to answer this question. Oogenesis is "diffuse" in nereids, meaning lack of any tissue confinement for the growing oocytes. Instead, oocytes are found distributed all over the body cavity (Fig. 2), and at sexual maturity the *Nereis* female is stuffed with full-sized oocytes. The coelomic fluid therefore is the medium surrounding and supplying the oocytes and we have, at various stages of oogenesis, gathered information about its metabolite composition. Moreover we have tried to imitate the composition of this fluid and succeeded in developing a culture medium for the oocytes and other coelomic cells of *Nereis* (Heacox *et al.*, 1983). As shown in Figure 3 oocytes and other coelomic cells can be isolated in tremendous amounts. Using the culture medium (with slight modifications), we are trying to elucidate the metabolic requirements and the incorporation capacities and kinetics of *Nereis* oocytes.

During gametogenesis, an essential part of the worm's biomass is converted into gamete biomass. This transfer of biomass implies a dramatic act of metabolic conversion, and since a major switch in the worm's metabolism is involved anyway in the onset of rapid oocyte growth, we are wondering whether or not this switch would be sufficient as a signal for the oocytes to engage in rapid growth. A significant change in availability of one or many metabolites in the coelomic fluid at the time of transition between slow and fast oocyte growth could be indicative of such a control of oocyte growth rate by a metabolic parameter. We are therefore particularly interested in the course of concentrations of various metabolites in the coelomic fluid around the period of transition from slow to rapid oocyte growth. However, whether or not a shift in concentration would be meaningful for the oocyte, depends on the absolute concentration of a metabolite in the coelomic fluid in relation to its parameters of uptake into the oocyte. Therefore, we need to learn both the course of metabolite concentrations and the susceptibility of the oocyte's uptake systems for particular metabolites during the time course of sexual maturation.

Which metabolites and metabolite concentrations are found in the coelomic fluid in the course of oogenesis? Glucose, the quantitatively dominant monosaccharide, free amino acids, with glycine, alanine and proline being quantitatively most important, and several nucleosides, mainly inosine and guanosine, are present throughout oogenesis (Hoeger and Kunz, 1993; Mahlein, 1993; Hoeger *et al.*, 1996).

For some amino acids and for two nucleosides, we have compared the parameters of uptake into the oocytes with their corresponding concentrations in the coelomic fluid during the time course of oocyte development. Two examples will be given below.

Inosine concentrations in the coelomic fluid range between 1 and 20 µM. In females of all stages investigated, these concentrations did not meet half-saturation conditions (apparent km= 40-140 µM; Hoeger and Geier, 1994). These findings suggest, that inosine supply is always below the maximal uptake capacity and may represent a limiting factor at least during the phase of rapid growth. Under physiological concentrations, cultivated oocytes incorporate this nucleoside at a constant rate for several hours. Under *in vitro* conditions (with 100 µM inosine), inosine uptake of oocytes isolated during the rapid growth phase exceeds the inosine uptake by oocytes of all other stages by an order of magnitude. Nucleoside precursors can be used for nucleic acid synthesis, and nucleoside supply appears especially significant for *Nereis virens* oocytes due to their high concentrations of DNA exceeding those of somatic tissues by several hundred fold (Sidorova, 1983). Still, however, it is not known if and to what extent nereid oocytes might in addition rely upon endogenous purine (and also pyrimidine) biosynthesis.

Alanine, one of the dominant amino acids in the coelomic fluid, is used in the oocyte as substrate of the oxidative metabolism and also serves as precursor for both gluconeogenesis and protein synthesis (F. Mahlein, unpublished results). Alanine concentrations in the coelomic fluid vary largely (0.1-1.1 mM). As found for inosine, the alanine uptake system is not saturated during the rapid growth phase considering the concentrations found in the coelomic fluid (Mahlein, 1993). Thus alanine, another compound of limited supply in the coelomic fluid, might likewise control oocyte growth rate. This situation of limited amino acid supply might be due to or amplified by competition by eleocytes (see below) incorporating amino acids as well (P. Heil, 1995).

However, the supply situation for growing oocytes has been found to be determined not only by low-molecular metabolites. Instead, the yolk protein precursor vitellogenin, a lipoglycoprotein, is provided by maternal tissues in many animals. Nereid oogenesis was for a long time taken as an example for the opposite, i.e. for anabolic autonomy. Unexpectedly, *Nereis* vitellogenin had been found as well in the coelomic fluid of *Nereis virens* (Fischer, 1979). We could prove that *Nereis* oocytes are capable of incorporating fluorescence-labelled (Fischer, 1984) or radioactively labelled authentic yolk protein (Fischer and Dhainaut, 1985; Fischer *et al.*, 1991) into their yolk bodies. Incorporation of this 320 kDa lipoglycoprotein (Fischer and Schmitz, 1981) is perfectly specific (Rabien, in Fischer and Hoeger, 1993). All these findings render the idea of autonomous yolk protein synthesis more than doubtful. In fact, Fischer and Rabien (1986) showed that *Nereis virens* oocytes are unable to synthesize their own vitellin.

We have therefore paid particular attention to the conditions for the uptake of yolk protein. Vitellogenin, a macromolecule produced specifically as a supply for the oocytes, appeared particularly suited to act as a component limiting and modulating oocyte growth. We have defined the uptake kinetics of the oocytes for vitellin during and after the period of rapid oocyte growth. The uptake was found to follow exactly simple saturation kinetics. Apparent K_m, standing for half saturation concentration of vitellin in the medium, was found a very reproducible parameter, ranging from 2.7 to 7.1 µg vitellin/ml in oocytes of medium size and from 34-40 µg/ml in oocytes of about 175 µm diameter (Fischer and Rabien, 1986; Fischer *et al.*, 1991). Thus, the incorporation of vitellogenin, certainly a major component of oocyte growth, seemed perfectly suited as a control instrument for the growth process directed by the vitellogenin supply.

We have, on the other hand, determined the vitellogenin concentrations available for the oocytes *in vivo* during the course of oogenesis (P. Heil, 1995). Using the ELISA technique of immunoreaction, a sample of 83 female *Nereis virens* as well as a number of worms still devoid of gametocytes in the coelom and

some males were assayed for vitellogenin in the coelomic fluid. Vitellogenin could be recorded in females exclusively and all of the females contained a remarkably high titer of vitellogenin (Fig. 1; stippled area). As uptake kinetics over a wide range of oocyte sizes indicate 90% uptake saturation at a concentration of <50 µg vitellogenin/ml, the concentrations found (about 70-560 µg/ml) in most females by far exceed those concentrations required for near-saturation conditions. Supply of vitellogenin thus does not appear to be the factor limiting the speed of oocyte growth in *Nereis virens*.

At this point, we can also consider the role played by the soma. Where in the soma and in which quantities does vitellogenin originate? In *Nereis virens* (Fischer and Rabien, 1986) and in *Perinereis cultrifera* (Baert and Slomianny, 1987) a specific type of coelomocytes, the "eleocytes" have been found to be the source of vitellogenin in the female. These rather large cells contain a voluminous vacuole and a small nucleus and are floating freely in the coelomic cavity along with the oocytes (Fig. 3-5). Lacking tissue integration of eleocytes is a favorable condition for taking them in cell culture, and indeed we have studied numerous biochemical parameters in freshly collected as well as in cultured eleocytes (Fischer and Hoeger, 1993; Hoeger, 1991; Hoeger and Kunz, 1993; Hoeger *et al.*, 1995).

Eleocytes are visibly engaged in the transfer of maternal biomass as they can be observed to phagocytose fragmenting muscle cells which appear in large numbers in the coelom during nereid sexual maturation (Geier, in Fischer and Hoeger, 1993). We have studied the course of vitellogenin secretion by eleocytes *in vitro* during the course of oogenesis (P. Heil, 1995). After the vitellogenin titers had been found unmodulated during oogenesis, however, the course of the vitellogenin secretion rates of the eleocytes during oogenesis came as a surprise. The rates of vitellogenin secretion take a strongly modulated course, with an average of 50 ng vitellogenin/µl PCVxhour in eleocytes from young females with oocytes of about 50 µm in diameter, increasing steadily with sexual maturation to a secretion of 170 ng and finally falling off to almost nil (7 ng) in eleocytes from mature females. Modulation in the rates of vitellogenin secretion by eleocytes thus coincides with modulations in the course of oocyte growth. Since modulated vitellogenin secretion is observed in cultivated eleocytes, i.e., in the absence of oocytes, the rates of vitellogenin secretion can neither be determined immediately by the presence nor by the growth stage of the oocytes. Therefore, eleocytes secretion rates obviously do not depend immediately on the vitellogenin demands of the oocytes, and, as shown above, oocyte growth rates are not controlled by modulated titers of vitellogenin supply from the eleocytes. Thus, the courses of both processes match each other but are not immediately causally linked. The nature of the element controlling and synchronizing both vitellogenin secretion rates and oocyte growth rates still needs to be defined.

Nereids are not unique with respect to their "monotelic" or "semelparous" mode of reproduction: all octopus and most species of squid, the lampreys and salmon of the genus *Oncorhynchus* reproduce only once in a lifetime as well and lampreys and salmon equally undergo a "catastrophic metamorphosis". In these organisms as well, the increase in egg biomass implies partial destruction of the maternal tissues. But in

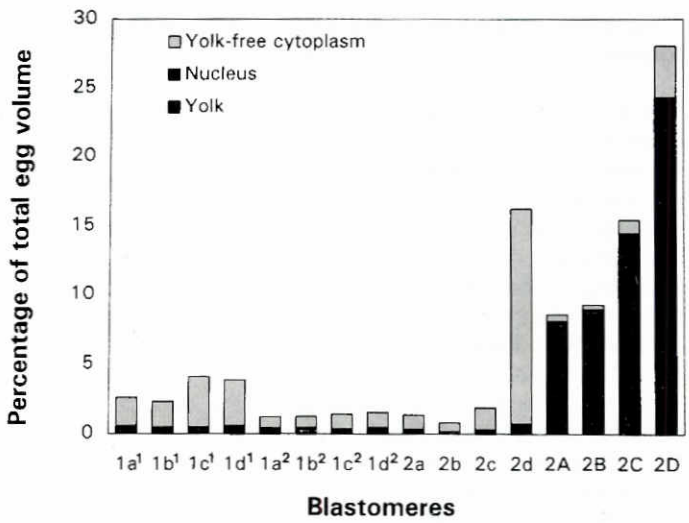


Fig. 9. Histogram with the morphometric data of the 16-cell embryo of *Platynereis dumerilii*. *The data were collected by planimetric analysis of serially sectioned embryos and are given as percentages of the total egg volume. The height of each column represents the volume of each blastomere (grey area, yolk contents; black area, nuclear volume; white area, yolk-free cytoplasm). Note that the major part of the yolk-free cytoplasm has been allocated to the 2d blastomere. The second-largest portion of the yolk-free cytoplasm is held by the 2D blastomere and is reserved for the formation of the 4d blastomere.*

none of these systems the metabolic trade-off and metabolic links are suited for analysis in such a way as in the nereids: while cephalopod and fish oocytes grow in tight connection with ovarian tissue, nereid oocytes are supplied and controlled via the coelomic fluid and are suited for cell culture studies. Moreover, even the somatic cells giving the nereid oocytes essential metabolic supply, the eleocytes, are free-floating cells whose physiology and metabolite export can be studied *in vitro*. We know that these cells besides secreting vitellogenin are exporting the nucleosides inosine and guanosine (Geier and Hoeger, 1993) which are incorporated by growing gametocytes, and we can safely assume that they are exporting lipids as well, since lipid contents of up to 300 mg/ml PCV in eleocytes of female *Nereis virens* during rapid oocyte growth are dropping down to some 50 mg/ml PCV in eleocytes of mature worms (Fischer and Hoeger, 1993). The perspectives of elucidating the metabolic pathways and trade-off between soma and growing oocytes in nereid sexual maturation, therefore, appear very promising.

Endocrinological ideas have dominated during many decades in the field of reproductive physiology, and polychaete reproductive physiology is no exception (see reviews and original papers in Fischer and Pfannenstiel, 1984). While there is no doubt that a brain hormone controls sexual maturation in nereids (Hauenschild, 1966; Porchet, 1970), from the existing experimental data it can still not be concluded which are the effector cells conveying the endocrine message to the physiological ensemble of the worm. Our cell culture studies on eleocytes and oocytes are likely to reveal whether these cells were primary targets of the endocrine signals or were controlled sufficiently well by metabolic links.

Cleavages and sorting out of plasms specify the histogenetic fate of embryonic cell lines

In the preceding section, we have described the construction of the nereid oocyte in terms of anabolism and have discussed the mechanisms controlling metabolic transfer of biomass from the soma into the growing oocytes. We will now consider the end-product of oogenesis, the fertilizable oocyte, from a quite different angle. We will adopt the embryologist's mode of view, regarding the fertilized spiralian egg as a cell entering cleavages programmed in space and time to give a set of cleavage cells (blastomeres). These are then determined for differing tracks of cell differentiation.

Already during the first cleavages in the nereid egg differences among the blastomeres in cell volume, nuclear volume and yolk content arise and are regarded as foreshadowing specific histogenetic potential of the cell lines to which they will give rise. According to a common notion of developmental biology, histospecific fates of cleavage cell lines can be traced back to specific regions of the uncleaved zygote (Anderson 1966). By an unreflected use of this "fate map" paradigm, physical carriers of specific fate information might then be inferred to reside in these specific regions of the uncleaved egg. We will present observations and experimental results indicating that in order to specify cell fate in early embryogenesis more subtle mechanisms are operating than the true transmission of specifying "determinants" already prelocalized in the egg.

The full-sized egg of nereids is heavily loaded with storage organelles. In a dramatic process of egg reorganization, the cortical granules sort out from the general egg cytoplasm during the 36 h preceding fertilization and form an extraordinary voluminous cortex (Figs. 6,7). After fertilization the egg discharges all of these cortical granules by exocytosis to give a wide jelly coat for the developing embryo. The period of exocytosis lasts 25 min, and so many cortical granules are extruded that the egg cortex is inferred to shed former organelle membrane to the amount of the six-fold of the egg surface (Kluge *et al., 1995*). Thus, the cortex of the egg is structurally reorganized before and immediately following fertilization. Therefore, it would be difficult to maintain a detailed pattern of developmental information (the existence of which may be suggested by the fate map paradigm) at the egg surface.

Cleavages in the nereid egg follow a strict spatial and temporal program. The geometry of cleavage planes and of the resulting blastomeres have already been carefully described by E.B. Wilson (1892, 1898) for *Nereis limbata*. Using computer-aided morphometry of fixed, embedded and sectioned embryos as well as observations of living embryos (Fig. 8), we have examined the course of early development of the closely related polychaetes *Platynereis dumerilii* (Dorresteijn, 1990) and *P. massiliensis* (Schneider *et al.*, 1992) as well as of *Pomatoceros triqueter* (Dorresteijn and Luetjens, 1994). We have thereby obtained precise data on the volumes and composition of all blastomeres at early cleavage stages and found only a limited variance in these parameters: The first cleavage in *P. dumerilii*, e.g., partitions the egg volume to 27% and 73%, respectively, with a standard deviation of only 1% of the egg volume.

Our results from these measurements confirmed the long-known impression of invariance in the cleavage pattern of so many spiralians. However, in addition our morphometric data reveal that in most cell divisions yolk and yolk-free ("clear") cytoplasm of the progenitor blastomere is not allotted to the daughter blastomeres in the same proportions as the volume. Instead, in the zygote as well as in many blastomeres a visible sorting process moves either sort of plasm into a position which is asymmetrical to the future cleavage plane. In *Platynereis*, the larger of the first daughter blastomeres comprises 73% of the zygote volume, but receives a disproportional 80% (±0.1%; n= 7) of the zygote's clear cytoplasm. This sort of programmed asymmetrical allotment of yolk versus yolk-free cytoplasm is enhanced during the following cleavages so that already at the 16-cell stage the volumes and cytoplasmic composition of the blastomeres are extremely disproportioned and diverse in the embryo of *P. dumerilii* (Fig. 9, after Dorresteijn, 1990). Exactly the same pattern of blastomere diversification is encountered in the much larger embryo of *P. massiliensis*, though at different proportions (Schneider *et al.*, 1992). In conclusion, diversity of the blastomeres in volume and contents is the result of two programmed processes, i.e. sorting out of plasms in an asymmetrical fashion and asymmetrical positioning of the cleavage furrow (possibly correlated with structural asymmetry of the cleavage spindle).

The question whether or not this blastomere pattern would be relevant for the imprinting of developmental fate on the cleavage cells is as old as modern embryology. However, a satisfactory answer to this question based on experimental results came up but slowly. In the embryo of *Platynereis dumerilii*, we have been able to alter the cleavage pattern by use of cytochalasin B (Dorresteijn *et al.*, 1987) or by centrifugation (Dorresteijn and Eich, 1991). We were able to dissociate the process of asymmetrical redistribution of the plasms from the process of unequal positioning of the cleavage furrow. Under such conditions, the experimentally changed four-cell stages showed an abnormal pattern of both blastomere size (Fig. 10 L_1, M_1) and distribution of plasms. Further development of such embryos resulted in very characteristic malformations of the Janus type (Fig. 10 L_2, M_2). Both the normal geometrical cleavage pattern and an undisturbed specific allotment of plasms to the blastomeres are therefore prerequisites for normal morphogenesis in nereids. Or, in reciprocal terms, the programmed cytological processes of plasmatic segregation and asymmetrical positioning of the cleavage furrow play a crucial role in determination of fate of the cell lines.

However, this conclusion does not tell us in which way these cytological parameters may act in order to produce such a determination. And in fact, this is the essential contemporary problem in the study of morphogenesis. We have tried to approach this problem in our system in several ways.

First, we have determined the temporal program of cleavages. The time schedule of cell cycles of blastomeres turned out to be difficult to determine by simply observing the formation of cleavage furrows. Furrow formation in nereid eggs starts from a single point of the future furrow circumference and propagates slowly. Instead, we therefore determined the starting-point of cell cycles by the disappearance of the nuclear envelope, e.g., the instantaneous prophase-prometaphase transition. Using this delimiter of nuclear events as a more accurate alternative for measuring cell cycle duration, we have found an interesting correlation. It has for long been postulated that yolk contents increase the duration of the cell cycle in a blastomere in com-

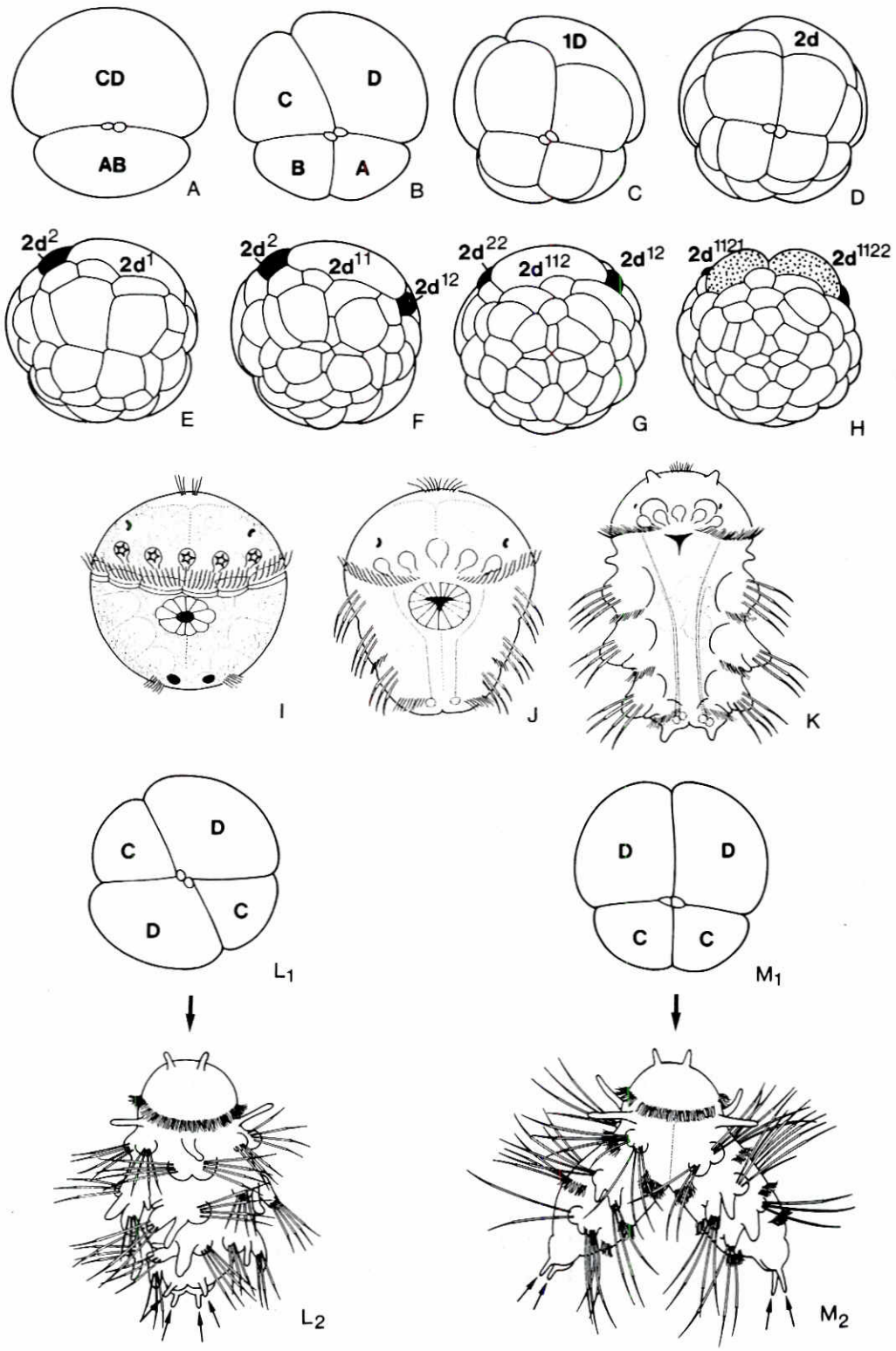


Fig. 10. Table of the development of *Platynereis dumerilii* under normal laboratory conditions in natural seawater at 18-19°C (A-K) and after experimental changes of the earliest cleavages (L1-M2). *The earliest blastomeres acquiring the competence to express a neural antigen or acetylcholinesterase upon cleavage-arrest in cytochalasin B are labelled black and dotted, respectively, and are denominated in the table of normal development using the nomenclature traditional for spiralian embryos. The five larval gland cells are labelled with star-symbols in the trochophore stage* **(I).** **(A)** *2-cell stage (2 h);* **(B)** *4-cell stage (2 h 30 min);* **(C)** *8-cell stage (3 h);* **(D)** *16-cell stage (3 h 40 min);* **(E)** *29-cell stage (4 h 30 min);* **(F)** *38-cell stage (5 h);* **(G)** *46-cell stage (5 h 40 min);* **(H)** *66-cell stage (6 h 30 min);* **(I)** *trochophore larva (24 h);* **(J)** *metatrochophore larva (48 h);* **(K)** *Three-segmented young worm (between 3-4 days).* **(L1)** *4-cell stage after experimental equalization of the first cleavage upon cytochalasin-B-treatment ("CDCD"-embryo);* **(L2)** *Three-segmented young worm developing after equalization of the first cleavage. The body pattern of such worms shows many duplications, like 4 rows of parapodia separating two dorsal from two ventral sides and four anal cirri (arrows);* **(M1)** *4-cell stage after centrifugation prior to the first cleavage ("CCDD"-embryo) and the resulting three-segmented young worm* **(M2).** *The body pattern of this worm also shows many duplications (four rows of parapodia and four anal cirri (arrows)), but here, the trunk is completely duplicated (forked) in the second and third segment.* **(A-K)** *after Dorresteijn et al. (1993);* **(L1-L2)** *after Dorresteijn et al. (1987);* **(M1-M2)** *after Dorresteijn and Eich (1991).*

parison with cells having less yolk (Schleip, 1929). Our morphometric analysis reveals that this hypothesis does not hold true in the *Platynereis* embryo. For instance, the extremely yolky blastomeres CD, D, 1D and 2D have the shortest cycles among the blastomeres of their respective cleavage stages. So, in fact, we could show that the "speed" of nuclear (as: cell) cycles is not proportional to the amount of yolk, but rather correlates with the absolute amount of yolk-free cytoplasm in a blastomere. Thus, the cell lines emanating from the D-line with the highest amount of yolk-free cytoplasm, i.e. the 2d- and 4d-cell line, exhibit the

most rapid rate of proliferation. It would be interesting to learn something about the quantitative correlation between the amount of yolk-free cytoplasm and the titers of cell cycle proteins which can be expected to control the duration of cell cycles in individual blastomeres. However, we must first explore the temporal structure of the cell cycles in the nereid embryo. At present, we know that no G1-phase exists in the early *Platynereis* embryo up to the 29-cell stage (C. Heimann, unpublished results). So, the lengthening of cell cycles in slowly proliferating cells must be ascribed to the titers of such factors controlling the duration of S-, G2- or M-phase, or alternatively, the timing of transition points.

In a different approach, we have studied the role which the partitioning of the zygote cytoplasm by new cell boundaries may play for the process of cell determination. Indeed, in the *Platynereis* embryo the specific pattern of cell boundaries is maintained in an extremely invariant way and is firmly correlated with the future pattern of cell fates. The future histogenetic fate of the offspring of each daughter cell is known as early as at the two-cell stage. A general concept predicts that progressive subdivision of zygote plasm by blastomere boundaries is necessary to stabilize the sorting out of plasms and of cell fates (Freeman, 1979). One would therefore like to know which steps of sorting out and cleavage were necessary to impose a specific fate upon a blastomere. This problem had been impossible to solve with the techniques available, such as the isolation of blastomeres, because in such experiments the majority of blastomeres appeared to remain in the undifferentiated state (with the exception of the trochoblasts; Costello, 1945).

We have adopted a technique described by Whittaker (1973) for ascidian embryos. In this approach, cell differentiation is considered not at the level of specific cell structures, but at the level of molecular differentiation, i.e. the appearance of cell-type-specific molecules. In normal development such cell-type-specific molecules cannot be expected in the blastomeres already. Differentiation of such molecular markers may be expected in cleavage stage blastomeres, however, whose further cell divisions have been interrupted and which survive until cell types have differentiated visibly in control embryos. In fact, our results show that experimentally retarded precursor blastomeres of some cell lines by that time may also already express the molecules specific for their offspring in normal embryos. Such molecules can thus be used as differentiation markers for (a) specific cell line(s).

In our experiments, we have used two sorts of molecules characteristic of certain nerve cells in the *Platynereis* larva: acetylcholinesterase (AChE) and a neural antigen detected by a monoclonal antibody. A different antigen could be detected in a group of five larval gland cells in the ventral head ectoderm (Fig 10I-K; Dorresteijn *et al.*, 1993). Using Whittaker's technique of cleavage interruption, we have studied the conditions required for the expression of these three differentiation markers (Dorresteijn and Graffy, 1993). In all cases, the differentiation markers became expressed in experimentally conserved cleavage stages, and in all cases, the expression of these markers was restricted to the respective precursor cells. With respect to the course of cell fate determination, several developmental principles were observed:

Competence for the expression of a histospecific marker may appear independent of cleavages, as in the case of the larval-gland-cell antigen, where even the cytoplasm of the cleavage-arrested, undivided zygote becomes competent after an appropriate lapse of time.

On the other hand, the precursor cell may not become competent or, in a sense, determined for a particular capacity until after a certain number of cleavages. The cleavage-arrested neural precursors, $2d^{2}$ and $2d^{12}$ (Fig. 10E,F), are the first to express the neural antigen. Another set of cleavage-arrested neurogenic cells, $2d^{1121}$ and $2d^{1122}$, are the first AChE-positive blastomeres (Fig. 10H). The cleavages of the cells 2d, $2d^{1}$ and $2d^{112}$, still negative for these markers convey marker expression to their respective offspring. We therefore conclude that these and the preceding cleavages serve to sort out components of the zygote plasm interfering with the marker expression and/or are required in order to generate a certain cell size.

As shown by Figure 10, however, these decisive divisions may act either in a symmetrical or an asymmetrical fashion: Only one of the two daughter blastomeres of the cells 2d and $2d^{1}$ (and in both cases the much smaller ones, $2d^{2}$ and $2d^{12}$) are becoming competent for marker expression; the decisive role played by the cleavage thus is asymmetrical, and the respective sister blastomeres $2d^{1}$ and $2d^{11}$ do not acquire the capacity for marker expression. In a different cell line, the histogenetic fate decision by a cleavage may concern both of the daughter cells, as is the case in the cleavage subdividing blastomere $2d^{112}$ into the cells $2d^{1121}$ and $2d^{1122}$ (Fig. 10G,H), a cleavage symmetrical both in geometry and fate determination (expression of AChE).

Competence for marker expression may continue to be transmitted asymmetrically in a sequence of cleavages: blastomere $2d^{22}$ solely inherits this capacity from cell $2d^{2}$, and this mode of fate-restricting cleavages was also observed for the larval-gland-cell antigen. In this case, the course of cleavages was found to restrict this competence from the uncleaved zygote to ever smaller portions of the embryo until finally the five larval gland cells were the only part of the organism capable of expressing this antigen.

In conclusion, sorting out of biochemical potential for the synthesis of differentiation markers and their progressive segregation are mechanisms conferring the capacity for the expression of histospecific molecules to the cells of an embryo. The results of our experiments all support the hypothesis that sorting processes and rearrangement of different sorts of plasm as well as a programmed subdivision of the zygote plasm by cell boundaries are essential for the imprinting of cell fate to the blastomeres or at least for directing competence for marker expression into specific cells of the embryo. Interference with cleavage geometry leads to malformations, and interference with the progress of subdivision of the egg plasm may inhibit the future expression of histospecific molecules. Thus, the early decisions about development in the nereid embryo appear to follow entirely a program of cellular epigenetic events. Our observations in living and fixed embryos, showing rearrangements of plasms and the way how cleavage geometry is brought about by the division apparatus are very suggestive illustrations for such an interpretation. It therefore appears desirable to learn more about the qualities of different sorts of plasms and about the question

whether perhaps the function of "determinants" is a matter of quantities rather than of qualities.

Conclusions

Nereid annelids range among the less known systems of developmental research, but appear attractive for the study of development for several reasons:

- In preparation for a single act of reproduction, the individual invests all of its resources into the production of gametes, thus becoming involved in massive catabolism of somatic biomass and anabolism for the benefit of the gametocytes.
- The gametocytes as well as the eleocytes (somatic cells playing a key role in metabolism) are free-floating cells within the coelom. They can be separately kept and studied in culture. Thus, control of oogenesis putatively exerted by selected metabolites, can be explored and might suffice as control element(s) for synchronized gametocyte differentiation. A ubiquitous controlling function of the well-known nereid hormonal system, postulated by earlier authors, thus appears not required by necessity. Conditions in the nereid system appear favorable for studying control of gametogenesis at the level of metabolism and cell physiology as a hitherto rarely considered alternative to hormonal control of oogenesis. Moreover, the conditions in nereid oogenesis enabled us to get a rather unique insight into the metabolism and anabolism of a growing animal oocyte.
- The cytoplasm of the full-sized nereid oocyte undergoes a rather rapid sorting process, ending up with a fertilizable oocyte in which the different storage organelles form several layers. The outermost layer, the cortex, is made up by extremely numerous cortical granules. Exocytosis of these granules after fertilization causes a profound structural reorganization of the egg cortex.
- We have found early development of nereids a very suited research object because of its invariant spatial plan and temporal schedule, because of its strongly polarized cleavage pattern and because of the transparency of the egg. Using computer-aided morphometry, we have therefore determined the geometry of the egg's sequential subdivision by cleavages, the sorting out of different plasms and the time schedule of cleavages in the embryos of a pair of sibling species. We have found rules being followed firmly.
- Disturbing the normal proportions among cleavage cells may cause characteristic malformations proving that an exact plan of plasmatic segregation and of cleavage geometry needs to be followed in order to guarantee normal development.
- Experiments in which we have interfered with the progressing segregation of plasms and with cleavages have shown that both of these processes are required for localization and realization of histogenetic potential. They also showed that such a localized determination is achieved by diverse mechanisms and follows diverse patterns: depending on the cell line, segregation of plasms and the occurrence of cleavages may or may not be required for the process of determination, and the state of determination during further cleavages may be transmitted symmetrically or asymmetrically (i.e., to only one of the daughter cells).
- Nereid embryos thus appear suited for studying the cell- physiological program and mechanisms which obviously direct the process of histospecific determination in this type of embryogenesis.

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