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Boveri's contributions to developmental biology – a challenge for today

KARL B. MORITZ[1]* and HELMUT W. SAUER[2]

[1]*Zoological Institute, University of Munich, Munich, Germany and* [2]*Texas A&M University, College Station, Texas, USA*

Introduction

Progress of knowledge is often the result of imaginative ideas of keen-witted minds. Research in the 19th century provided the Biological Sciences with three fundamental ideas which fruitfully influenced each other and radiated in almost all fields of research. They were germinal to all modern biological thought. They dominate all new conceptions in Biology, and unify this science in spite of its complexity. The first idea conceived the cell as the smallest autonomous unit of life. According to this concept, cells not only constitute the body of bacteria, protista, and complex higher organisms but they serve also as vehicles of propagation such as spores, sperm and eggs. The most important conceptual advance of the Cell Theory was the insight that new cells originate from old ones. It is expressed in the apodictic formula of Rudolf Virchow: 'Omnis cellula ex cellula'. The second idea is substantiated by the Mendelian paradigm. According to Mendel's idea, heritable traits are based on units, later called genes. These are permanent, individual particles that are transmitted from one generation to the next through the reproductive mechanism. The most seminal concept was that these units come in pairs (alleles). Variant alleles - Mendel's 'differing elements' - coexist in hybrids and are transmitted alternatively and in a single-copy state to the next generation. Thus, the principles of segregation and independent assortment were established from the combinatorial behavior of genes (Mendel, 1866). The third idea is already defined by the title of Charles Darwin's famous book 'On the Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life' (Darwin, 1859). Central to Darwin's thinking was to reject the essentialistic view of unchanging species. Thus, species were seen as the targets of a continuous experiment of Nature which we observe as the process of selective adaptation.

New paradigms in Science are sometimes ignored by the Scientific Community, more often misunderstood and always confronted with critical objections. Darwin was fully aware of the central open questions of his theory of 'descent with modification'. For example, the sources of biological variation and the principles of transmission genetics were unknown. He postulated an obscure pangenesis theory of inheritance. The Darwinian concept of evolution was a challenge for the young Cell Theory. August Weismann, after he had rejected all mechanisms compatible with the concept of instructive adaptation postulated the cellular continuity of the 'germ plasm' as a separate entity from which the mortal somatoplasm is derived generation after generation. He later identified the germ plasm as chromosomes, i.e. the germline genome of organisms (Weismann 1892).

In this classical period of Biology Theodor Boveri (1862-1915) (Fig. 1) began his scientific career. In 1885 he received his medical doctorate under the supervision of Carl v. Kupffer. In the same year Richard Hertwig took over the directorship of the Zoological Institute in Munich, and Boveri moved over from the anatomy department to him. Thus, in his intial research Boveri was fortunate to have one of the leading masters in Cell Biology at his side. Through the seminal work of Edouard van Beneden, Boveri was led to the Ascaris egg, and through the experimental work of Oscar and Richard Hertwig he became acquainted with his other experimental system, the sea urchin egg. Under the auspices of R. Hertwig, Boveri obtained his habilitation. His earliest publications already won him a prominent place among the leaders of cytology. At an age of 30, Boveri accepted the position as director of the Zoological Institute in Würzburg, and he remained in Franconia until his untimely death in 1915. In 1897

***Address for reprints:** Zoological Institute, University of Munich, Luisenstraße 14, D-80333 Munich, Germany. FAX: 89.5902450.

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Th Boveri.

Fig. 1. Theodor Boveri (1862-1915). *Photograph courtesy of Prof. Klaus Sander.*

he married his American PhD student Marcella O'Grady. Their only child Margret became internationally known as a political correspondent and writer.

According to Boveri's (1910b, p.1) own words, he wanted 'to analyse those processes whereby a new individual with definite characteristics is created from parental generative material'. In these modest terms he delineated one of the most important problems in biology. Two influential books in the field of developmental biology, namely 'The Cell in Development and Heredity' by Edmund B. Wilson (1925) and 'Experimentelle Beiträge zu einer Theorie der Entwicklung' by Hans Spemann (1936) may reveal the impact of Boveri's leadership in the field and of his personality: both were dedicated by their authors to Boveri's memory. According to Wilson 'Boveri's works are milestones of progress' and 'his writings will long endure as classical models of conception and execution' (Wilson 1918, p.67, p.69). This essay presents evidence that his most important concepts of the cell-division cycle and of the mechanisms of cell differentiation can still help to orient modern developmental biologists in their endeavors to unravel the relevant essential processes at the molecular level. However, Boveri's acute logic experimental operations and prescient insights may have not had the impact they deserve. Instead they were mostly ignored or completely misinterpreted.

Boveri's contribution to cell-division cycle concepts

The comprehensive cell studies of Boveri revealed to him cyclical processes during cleavage divisions in early embryos which are of significance for the reproduction of cells in general and – surprisingly – for cell-fate specification in development as well. A major advancement in his understanding of the cell-division cycle was the discovery and distinction of two cycles: the chromosome cycle and the centrosome cycle. Moreover, events commonly associated with fertilization also gave new insights into the control of the cell-division cycle.

The chromosome cycle

Although it was generally accepted in Boveri's time that cells are generated by binary fission of a mother cell, it was not clear how chromatin, Weismann's germ-plasm, that is located in the cell nucleus is transmitted to the daughter nuclei such that both are genetically identical to the nucleus from which they arise. The main problem concerned Walther Flemming's 'nuclear metamorphosis', i.e. the transformation of a nucleus into dancing mitotic chromosomes and back into interphase daughter nuclei. The change of chromatin into threads inspired the term 'mitosis', but did not explain how these elements, already anticipated as the vehicles of hereditary traits, are precisely reproduced and distributed to daughter cells.

Boveri solved this problem in principle by demonstrating that chromosomes are permanent organelles which are condensed in mitosis and dispersed during interphase. But besides his observations concerning continuity and individuality of chromosomes, important arguments gathered in support of the Chromosome Theory of Inheritance, Boveri gave a truly modern description of the establishment of the mitotic apparatus (MA) and the interplay of its components to accomplish the distribution of daughter chromosomes to opposite poles.

From detailed observations of cleavage cycles in Ascaris (*Parascaris spec.*) and sea urchin embryos, including multipolar mitosis, half spindles, and monopolar mitosis – which he first observed as spontaneously occurring abnormalities and later was able to induce experimentally – Boveri (1888b, 1904a, pp. 23-25) arrived at three rules of chromosome distribution (Fig. 2).

1. In mitosis, chromosomes are bipartite and display a 'sideness' such that astral microtubles (Mt's, the 'radii' of the astropheres) can connect only at the pole sites. This rule includes (i) the concept that a mother chromosome splits into two and only two chromatids which are consistently distributed to opposite poles, and (ii) a vision of the not yet discovered kinetochores, at opposite sites of chromatids which he first termed the chromosomal narrow sites. With respect to the special attachment sites of spindle fibers, Boveri distinguished two different types of chromosomes, equipped with either a localized centromere (sea urchin, see Fig. 3) or a diffuse centromere (Ascaris, see Fig. 2). Nothing is known about those factors which establish the chromosome sideness, i.e. the handedness of sister chromatids along their entire length.

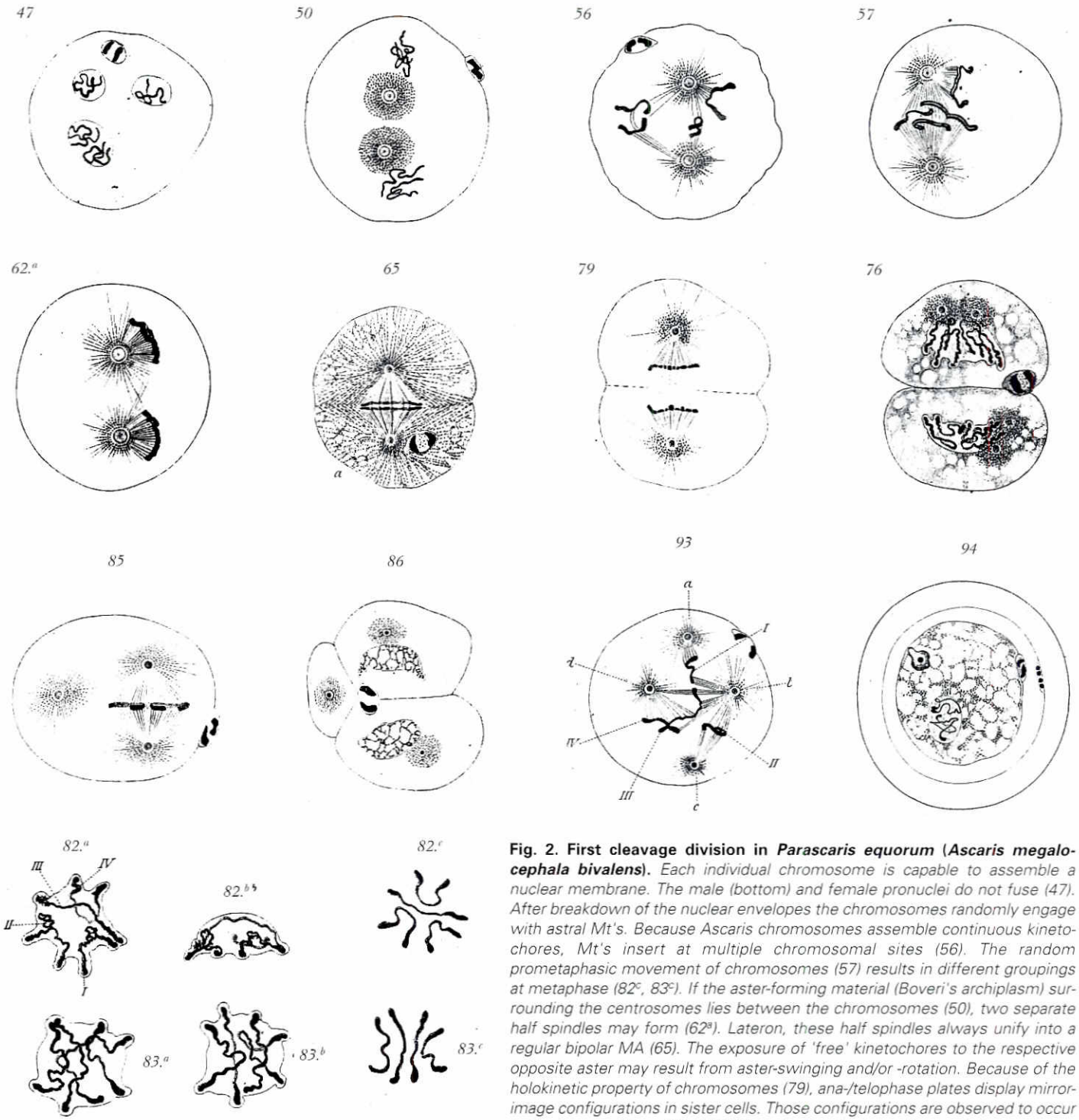


Fig. 2. First cleavage division in *Parascaris equorum* (*Ascaris megalocephala bivalens*). *Each individual chromosome is capable to assemble a nuclear membrane. The male (bottom) and female pronuclei do not fuse (47). After breakdown of the nuclear envelopes the chromosomes randomly engage with astral Mt's. Because Ascaris chromosomes assemble continuous kinetochores, Mt's insert at multiple chromosomal sites (56). The random prometaphasic movement of chromosomes (57) results in different groupings at metaphase (82ᶜ, 83ᶜ). If the aster-forming material (Boveri's archiplasm) surrounding the centrosomes lies between the chromosomes (50), two separate half spindles may form (62ª). Lateron, these half spindles always unify into a regular bipolar MA (65). The exposure of 'free' kinetochores to the respective opposite aster may result from aster-swinging and/or -rotation. Because of the holokinetic property of chromosomes (79), ana-/telophase plates display mirror-image configurations in sister cells. Those configurations are observed to occur in the next prophase (76, 82ª-83ᶜ), indicating the persistence of chromosomes as individuals during interphase. In tetrapolar mitosis sister chromatids exclusively engage with only two asters (93). Breaks and bridges of chromatids do not occur at anaphase. Obviously, the current concept according to which the direction of Mt growth determines the Mt capturing capability of kinetochores cannot sufficiently explain Boveri's rule of the engagement of one aster per one chromatid, first established on continuous kinetochores. Formation of the cleavage membrane occurs midway between the centers of the aster pairs (65, 85, 86). This is inititiated by furrowing at the cell surface along the meridian equidistant to the centrosomes. This meridian defines the position of the contractile ring (unknown to Boveri). Accordingly, in tetrapolar mitosis there are six segments of those meridians, which form four spherical triangles on the cell's surface. Hence the cell cleaves simultaneously into four daughter cells (For simultanvierers see Fig. 9). In case of fertilization with an inactive sperm (94.), nevertheless all events of normal fertilization may occur, like meiosis and polar body formation, elevation of the fertilization membrane, and compaction of the egg as indicated by the perivitelline space. After chromatin replication the female nucleus entered mitosis albeit an 'achromatic apparatus' is absent. From Boveri (1888b).*

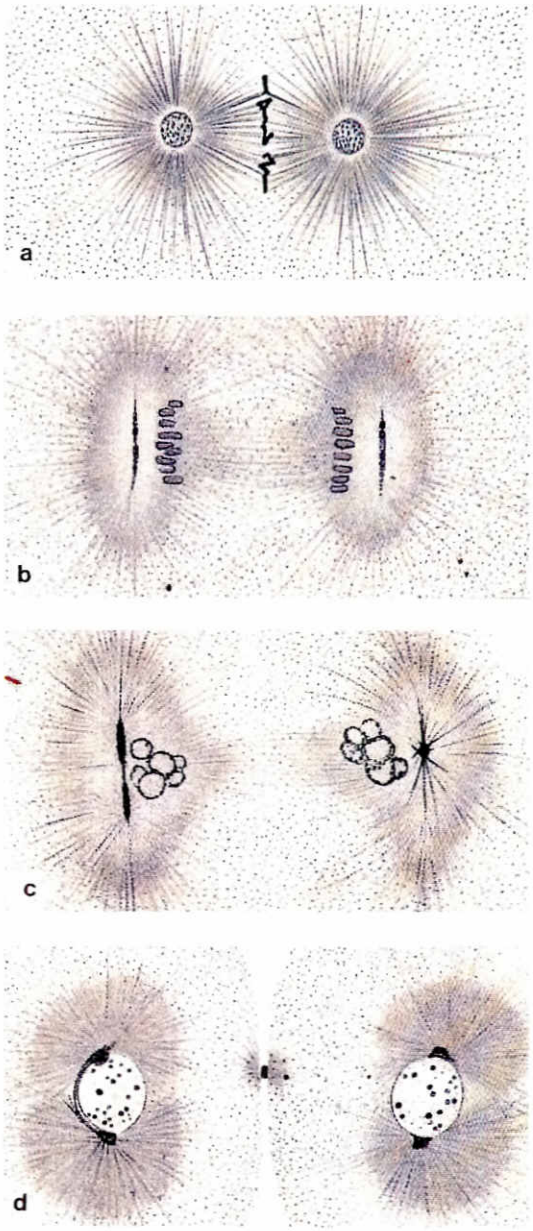


Fig. 3. First cleavage mitosis of *Paracentrotus lividus*. According to Boveri centrosomes are 'cycling organelles'. *When they contain centrioles, these 'passengers' duplicate prior to centrosome growth. At metaphase* **(a)** *centrosomes attain maximal size and have spherical appearance. During formation of karyomeres* **(b)** *the centrosomes flatten into disc-shaped bodies. Concomitantly with aster fission,* **(c)** *centrosome splitting occurs, drawn at two different views. Separation into two daughter centrosomes* **(d)** *occurs on the nuclear envelope by centrosomal compaction. In the left blastomere the daughter elements are still connected by a stalk. Although centrosome separation occurs regularily on the nuclear envelope in sea urchins, nevertheless nuclei are not necessary for centrosome cycling. Boveri demonstrated that centrosome cycles occur normally in enucleated cells. From Boveri (1900).*

2. If one Mt from one aster has been connected to one side of a mitotic chromosome, i.e. to one chromatid, other Mt's from the same aster must connect with the same side. This property is by no means trivial because it holds also for continuous kinetochores, as first observed in the case of Ascaris germline chromosomes by Boveri (Fig. 2: 57, 93). Are there adaptors in the spindle which cause chromatid orientation towards a single aster? These adaptors would have to interact with factors establishing the bipartite structure of replicated chromsomes.

3. If one sister chromosome has been connected to one pole, Mt's from another pole can only connect with the other chromatid (Fig. 2: 93). – Albeit the Ascaris chromosomes contain numerous MT attachment sites, normal chromatid segregation consistently occurs not only at bipolar but also at multipolar mitosis (see also Fig. 9: 13d).

Boveri (1904a, p.18) also realized that the chromosome cycle includes the process of chromatin duplication during interphase. From his work on haploid, diploid, triploid, tetraploid, and aneuploid nuclei in several experimental sea urchin embryos (see Fig. 5), he (1905, p. 60) deduced a precise correlation between the number of chromosomes, or the amount of chromatin and nuclear size (more accurately the nuclear surface). This correlation he applied to document "proportional nuclear growth" and concluded that chromatin doubles once before the respective cell enters mitosis. For the chromosome cycle Boveri defined three essential events: chromatin reproduction in the resting period (interphase), the splitting of chromosomes into daughter elements (chromatids) during chromosomal condensation, and chromatid distribution at anaphase. In modern terms, the replication and assembly machinery is active at the first period, at the second mostly DNA topoisomerases and those enzymes work which are involved in the modification of chromosome-integral proteins, and at anaphase activities are induced that are localized in the kinetochores and cause chromosomal spindle fiber degradation. The full implication of Boveri's concept of the chromosome cycle becomes only apparent in considering cyclical events in the cytoplasm. To him the passive nature of chromatin in the nuclear cycle was selfevident what is expressed in the phrase 'The nucleus, i.e. the chromatin,does not divide, it is divided.'

The centrosome cycle

Boveri (1887a) was not the first to see the centrosome – independently E. van Beneden and A. Neyt observed the 'corpuscule central' inside the asters – but he recognized its role to the extent that is only now being appreciated. He clearly defined the centrosome, a pair of centrioles surrounded by a special material, which is capable to assemble a sphere of archiplasm containing all those components involved in the transient generation of an astrosphere (Fig. 2: 50). Boveri (1900) definitely showed that centrosomes are cell-autonomous and single-copy organelles (Fig. 3).

He forcefully argued that 'the centrosome functions as the proper cell division center' (1887b, p. 154). Its organizing role in mitosis, exerted throughout the cell cycle, was deduced from three observations: (i) its capability to reproduce and to divide once per cell cycle, (ii) a tendency of moving apart of the daughter centrosomes during interphase, normally into defined cell regions, or at equidistant positions at multipolar mitosis, and (iii) their functional equivalence.

Deep insight into the centrosome's role in the establishment of the MA, especially for the force-generating activity was gained from the analysis of monasters, i.e. monopoles, that result from

blocking centrosome division but allow all other cellular activities in preparation for mitosis. Monasters are 'spheres of influence' over a large domain of cytoplasm. If nuclei are present, the monaster produces a 'half spindle' on which chromosomes are aligned on a curved metaphase plate as shown in Figure 2: 62.[a] for the purivalent chromosomes of *P.equorum* (n=2) which contain several heterochromatic (HET) blocks. In *P. univalens* (n=1) the large compound chromosomes are composed of terminal HET blocks and an euchromatic intercalary segment which contains the somatic genome equivalent (Fig. 7:1.). The HET blocks consist almost entirely of two satellite DNAs each of which is composed of one short unit repeat at high copy number, and the canonical Ascaris repeated telomeric sequences. Only the intercalary segment assembles a continuous kinetochore, the HET blocks are acentric (Fig. 7:11a). Nevertheless the large HET blocks take part in formation of the 'chromatic plate' at metaphase (Fig. 7:1). The force-generating components of the achromatic apparatus which accomplish the transport of the long chromosome arms away from the asters and maintain alignment of the chromosomes at the metaphase plate still await molecular description.

From the three rules of chromosome engagement with asters and the dynamics of the monopolar mitosis, Boveri arrived at the far-reaching conclusion that the typical bipolar spindle is the fusion product of two half spindles held together by the sum of bipolar chromosomes, i.e. chromatids facing to opposite poles (1887a, p.79). The identical appearance of half spindles – as either solitary structures or parts of bipolar and multipolar MA's, respectively – at metaphase, characteristic of their minimal aster-to-metaphase plate distance, indicated to him a stage of equilibrium (Boveri's term). In dispermic sea urchin eggs Boveri observed formation of two bipolar MA's. For this 'double spindle' type he emphasized the observation that the pairs of asters engaged with chromosomes come closer together than those not connected by chromosomes. Therefore, chromosomal forces appear to counteract aster repulsion during metaphase plate formation.

At anaphase, initiated by a still mysterious switch, two identical sets of chromosomes are distributed towards opposite poles. Boveri (1888b) observed that this movement is accomplished by two different activities: (i) the shortening of Mt's (Boveri erroneously postulated fiber contraction.) which connect the asters to the cortex of the opposite cell poles, and (ii) of spindle fibers, i.e. Mt's which attach at kinetochores (Fig. 2: 79). In monopolar mitosis chromosomes move also towards the single aster at anaphase. However, chromatid separation does not occur. The chromatids apparently segregate at interphase since twice the number of chromosomes enter the following mitosis. In multipolar mitosis chromosome engagement with pairs of asters occurs at random (Fig. 2: 93). Therefore, the poles receive different aneuploid complements of chromatids in most cases (see Fig. 9).

Recently, Daniel Mazia (1987) in a review "The chromosome cycle and the centrosome cycle in the cell cycle" resurrected the three rules of Boveri concerning the establishment of the MA, 'not as a genuflection of the past', but because they are 'completely valid statements'. Likewise, most details of the organizer function of the centrosome are not yet understood. Mazia also accepts Boveri's notion of two half spindles forming the bipolar MA, as to him the rigid physical arrangement of sister kinetochores is a sufficient precondition to engage with only one aster per kinetochore. Mazia also notes that E.B.Wilson, in his book 'The Cell in Development and Heridity' (1925) although incorporating most details of Boveri's work on mitosis, fails to refer to his demonstration that centrosomes are permanent cyclical organelles, acting throughout the cell cycle. Only recently has this view come into focus along with genetic studies and attempts to reproduce the centrosome cycle in cell free systems.

Boveri (1896) succeeded with two elegant experiments, demonstrating the autonomy of the centrosome and chromosome cycles by separating these two organelles in the first cleavage division of the sea urchin egg. As a result of induced mitotic nondisjunction of all chromosomes one blastomere contained only one centrosome while the other contained all chromosomes besides the sister centrosome. In the enucleated blastomere the centrosome and its descendants continued cycling by synchronous reproduction, followed by periodic aster formation in synchrony with division of the sister cell and its descendants. This finding of Boveri has been worked out by his wife Marcella Boveri in a fine cytological analysis on stained sections of single sea urchin embryos (1903). In a complementary experiment Boveri fragmented activated (fertilized) eggs and isolated those fragments which contained the female pronucleus but did not contain the sperm-derived nucleo-centrosomal apparatus. In these egg-fragments he observed several rounds of breakdown of the nuclear membrane followed by nuclear growth, i.e. chromatin doublings. From these results, reflecting on a 'dualism of the phenomena of nuclear division', Boveri concluded that the two autonomous cycles are normally coupled by the cytoplasmic state that is itself oscillatory and set in motion at fertilization (see also Fig. 2:94).

A further conclusion was that both chromosomes and centrosomes must collaborate in cell division, at least in the sea urchin, since cells missing either one of the two organelles are unable to undergo cytokinesis. A guiding role of the centrosome for cell division can be seen from the fact that a cell divides into as many cells as there are asters present (Fig. 2: 85, 86). In addition, cleavage planes are determined by centrosome position: Furrowing consistently occurs at planes equidistant to the centers of asters (Fig. 2:65). Thus, centrosomes are always distributed to the daughter cells, and cleavage always occurs in the plane of the metaphase plate, even if the (bipolar) MA is asymmetrically positioned in the cell. Monaster cells fail to divide, but at the cell surface furthest from the aster exovates appear, indicating the transient formation of a contractile ring at that site (1903).

Boveri (1896) suggested that Mt's radiating from the surface of centrosomes (see Fig. 3) are necessary for cell division (Fig. 2: 85). After cold or pressure treatments of sea urchin eggs he observed disassembly of astral Mt's. Placed back to normal conditions the Mt's reappeared immediately. However, after induced Mt decay at a very late mitotic stage, the centrosomes were no longer able to reassemble Mt's. Concomitantly, the cell failed to divide. These findings indicate the dynamics of Mt-nucleating activity of asters. Boveri postulated a critical period in the centrosome cycle which is defined by its aster-forming potential. This period becomes terminated prior to the end of the cell cycle, apparently by a sudden event which alters the cytoplasmic milieu.

Boveri's most fundamental insights in the mechanism of cytokinesis in animal cells can be summarized as follows:
1. Any segment of the cell surface can participate in furrow formation. Evidence for this statement are, for example, the results of Boveri's many centrifugation experiments in Ascaris (1910a; see Fig. 11).
2. Asters inhibit furrowing, i.e. the formation of cortical contractile structures, in their neighboring cell-surface domain. – In the Ascaris triaster egg-cell of Fig. 2:86 the extra aster prevented completion of cleavage along the plane of the metaphase plate.
3. Asters cooperate in pairs in furrow formation. In triaster cells three pairs of asters (Fig. 2:85,86) and in tetraster cells six pairs of asters cooperate in establishing the cortical contractile structure. Nevertheless, Boveri observed cleavage of Ascaris eggs into three cytoplasmic segments after the bipolar MA had been shifted to an eccentric position in the cell: cleavage resulted in the formation of two daughter cells and a persistent exovate carved out of the egg part furtherst from the MA (Fig. 10). From this type of exovate formation (in the course of cleavage) Boveri (1910a) suggested that each aster is capable to stimulate furrowing at the margin of its sphere of influence.
4. Because cells containing several asters initiate furrowing simultaneously at multiple surface sites, the transient signal inducing cleavage appears to spread rapidly through the cytoplasm.

To conclude: the achromatic apparatus dictates the formation and positioning of the cortical contractile structure. The activity of this structure causes furrowing. Boveri's observation (1896) that the chromatic plate contributes to the formation of a persistent cleavage membrane points to the enigmatic process by which cytokinesis is completed. Chromosomes appear to deposit certain factors in the equatorial plane. These factors would participate in the formation of a functional diastem bridging the edge of the cleavage furrow, and thus would assist the fusion of the plasma membrane which definitively separates the cell into daughter cells.

Boveri (1892) proposed uniparental inheritance of the centrosome. In Ascaris the maturation divisions in the oocyte occur at 'anastral' spindles (Fig. 4). Therefore, he suggested centrosome loss during growth of the oocyte (Fig. 2: 94). On the other hand, his cytological analysis of spermatogenesis revealed the presence of centrosomes. In the sea urchin egg the rudimentary oocentrosome apparently becomes inactivated after maturation. Earlier it was suggested by Hermann Fol that the 'quadrille of centrosomes', i.e. four centrosomes, derived from both germ cells initiates the first cleavage cycle of the zygote. Boveri thoroughly de-romanticized this dance as he did the dance of the chromosomes during 'nuclear metamorphosis'.

In his heterosperm insemination experiments done with several sea urchin species, Boveri (1889) observed development of the egg into hybrid larvae. Therefore, the sperm-derived centrosome is capable to work in a foreign cytoplasm. This interspecies cooperation strongly argues for a high conservation of the essential constituents of centrosomes during evolution. Mainly from his observation that centrosomes are capable of undergoing numerous rounds of reproduction even in enucleated blastomeres (see above), Boveri suggested that mature egg cells contain a considerable store of centrosome constituents. Chromosomal centromeres, on the other hand, do not work in all hybrid crosses. For example in the cross *Sphaerechinus granularis* (♀) x *Paracentrotus lividus* (♂) the paternal chromosomes are propagated normally in the *Sphaerechinus* cytoplasm. In the reciprocal cross almost all sperm chromosomes become eliminated already at the first cleavage mitosis, apparently because of malfunction of their kinetochores at anaphase. This observation has been made by Fritz Baltzer, a student of Boveri.

Boveri (1910a,b) has contributed two original concepts concerning embryonic cell-cycles. First, he showed that these cell cycles are intimately involved in the specification of cell fates. The seminal discovery of a developmental timing-device that calls for a cellular memory coupled to the cytoplasmic oscillatory system which coordinates the chromosome and the centrosome cycles, was made with sea urchin embryos. By shaking at sensitive stages of the first cleavage cycle in sea urchins, centrosome division could be blocked transiently which, in turn, prevented cell division. In normal embryos a special cell-type, the micromere, is formed at the vegetal pole after four cleavage cycles (Fig. 6). If the first, or the first and the second cell divisions are skipped by preventing centrosome reproduction, the egg undergoes 1/2-cleavage (its cleavage pattern corresponds to that of a blastomere of the 2-cell stage) or a 1/4-cleavage, respectively. Hence, micromeres still form at the correct time, but in 8- or 4-cell embryos, respectively, instead at the typical 16-cell stage. This phenomen has been termed 'partial cleavage', and Boveri's findings on partial cleavage have been confirmed and extended by Hörstadius (see Hörstadius, 1973). Obviously, a developmental timer, once set in motion at fertilization, keeps running and counts of cleavage cycles, even in the absence of cytoplasmic segmentation, i.e. cytokinesis, but the molecular nature of the timer which must involve some discontinuous alterations in the cell's state has remained elusive to this day.

Second, from a brilliant cell-lineage analysis of experimental Ascaris embryos Boveri (1910b) deduced that cell cycles are 'activation cycles'. Boveri's ingenious concept of segregative cleavage divisions, according to which the MA is capable to sense the polar organization of a cell and is forced to orient along the axis of polarity, has remained unnoticed for almost 100 years (see chapter 'Ascaris studies').

Boveri's theory of fertilization

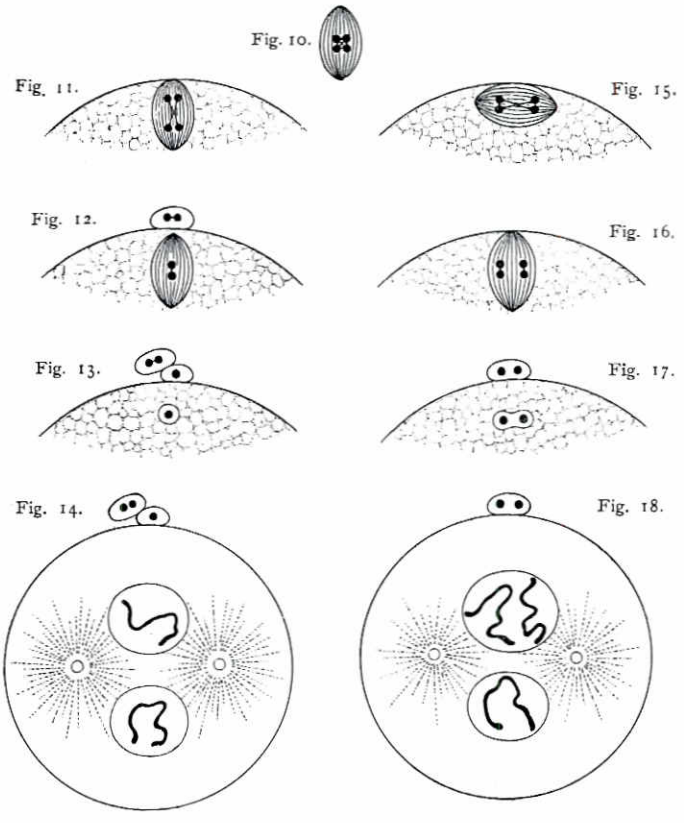
From the observation that traits of both parents are inherited by their offspring and O. Hertwig's demonstration of the fusion of male and female pronuclei in the process of fertilization, it was generally accepted that karyogamy is the essential event in the initiation of a new life cycle. Not so according to Boveri's logic. Always turning to the earliest event discernable, for Boveri (1892, 1907) fertilization primarily removes a blockade of cell division that has kept the growing oocyte and the mature egg-cell (the single cell-type in plants and animals abrogating R. Hertwig's rule of a constant nuclear-cytoplasmic ratio) from beginning a new life cycle. He considered the import of the paternal centrosome as the essential missing step that enables the egg to divide.

Only monospermy allows the generation of a bipolar MA followed by binary fission of the egg and its descendants. Consequently, a barrier to polyspermy must go hand in hand with egg activation, initiating the cytoplasmic cycle that controls both the chromosome and centrosome cycles (see above).

By two kinds of experiments, Boveri showed that fusion of the nuclei from opposite gametes – although a consequence of fertilization – is not essential for development since either nucleus is dispensable. First, by shaking sea urchin eggs, fragments are generated that lack the nucleus. These enucleated eggs can be fertilized ('merogons'), and the sperm nucleus (besides the paternal centrosome) is sufficient to guide their normal development. Secondly, by treating sperm with chemicals, Boveri (1888a) succeeded in triggering 'partial fertilization'. This means that the sperm nucleus was unable to take part in development. However, its centrosome by providing the MA organizer for the female nucleus still allows development. Obviously, the two nuclei are equivalent, and sea urchin development does not require karyogamy. This is further shown in cases of 'delayed fertilization' where the inactivation of the sperm was only transient. After completion of the first mitotic division of the egg nucleus, the sperm nucleus had recovered, and it fused with the nucleus of the daughter cell to which it was partitioned. This embryo develops normally into a pluteus. Its specific mosaic character, one side haploid and the other diploid, indicates that the first cleavage plane determines the median axis of the bilateral pluteus in the undisturbed embryo.

This careful observation, in accord with the idea of primacy of egg activation over nuclear fusion, allowed Boveri (1915) to explain a mysterious case in honey bees, the famous 'Eugster gynandromorphs'. These bees display traits of either their (Italian) mother or their (German) father over different parts of their body. One explanation of these gynandromorph mosaics was offered by Thomas H. Morgan. He argued that the mosaics were caused by two sperms accidentally fertilizing one egg. Consequently, half of the mosaic bee was diploid, descended from the fusion product of one sperm nucleus and the egg pronucleus, the other half was haploid and derived from the extra sperm. However, there was a problem with Morgan's interpretation. The haploid male portion should look like its (German) father. But it looked like its (Italian) mother. Boveri, remembering his analysis on mosaic sea urchin embryos caused by delayed karyogamy, could offer a definitive explanation: the haploid male domains of the mosaic bees are derived from precocious division of the egg nucleus, and as such should display matroclinal traits, while the diploid female domains should also express patroclinal characters. It seems that the resolution of the gynandromorph controversy between two great geneticists is a direct outcome of Boveri's theory of fertilization as primarily de-repressing cell cycles and secondarily uniting two parental genomes.

It is interesting to note that events surrounding egg maturation and fertilization have contributed immensely to the current generalized view of eukaryotic cell-cycle controls. It is now accepted that MPF (maturation and mitosis promoting factor) may be part of the common mitotic oscillator and contain a cyclin-dependent kinase, encoded by a gene shared by all eukaryotic organisms. Moreover, Boveri's concept of egg activation as release from a blockade is being clarified by the analysis of CSF (cytostatic factor), and the implication of a rather 'non-informational' small molecule (free calcium ions) in providing a link in the coupling of the centrosome and the chromosome cycles to fertilization as an intracellular messenger. The visionary conclusion of Boveri that the centrosome is the master organizer of cell division still awaits molecular analysis.



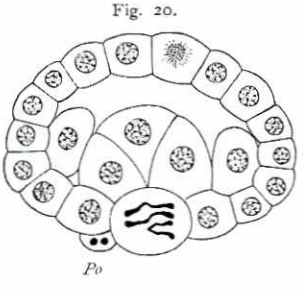


Fig. 4. Meiosis in *Parascaris univalens* and continuity of chromosomes. *The fertilized oocyte which contains a single tetrad undergoes two maturation divisions (Figs. 11-14, Ascaris type of meiosis). Normally, from each pronucleus, one chromosome enters mitosis. In case of paratangential spindle orientation (Fig. 15) extrusion of the polar body I fails to occur (Fig. 16). Consequently the female pronucleus contains two chromosomes (Fig. 18). The triploid chromosome set (two female chromosomes, one male chromosome) at later cleavage (Fig. 20) corresponds to two chromosomes in polar body II (P_0) (see Fig. 17), and absence of polar body I (Fig. 16). The barrel-shaped meiotic spindles indicate loss of the centrosome-organizing center during oogenesis. According to Boveri, in nematodes and sea urchins the active centrosomes are paternally transmitted. From Boveri (1904a).*

Chromosome theory of inheritance

One might ask what was known about the elements of inheritance and the behavior of their vehicles during fertilization at the time when Gregor Mendel designed his imaginative crosses in garden peas. In the 'Concluding remarks' of his brilliant treatise entitled 'Versuche über Pflanzenhybriden', for Curt Stern 'a triumph of human mind', Mendel (1866, p. 58) stated: 'According to the view of famous physiologists, in phanerogames propagation is initiated by the union of one germ cell and one pollen-grain cell into one single cell which develops into an individual organism

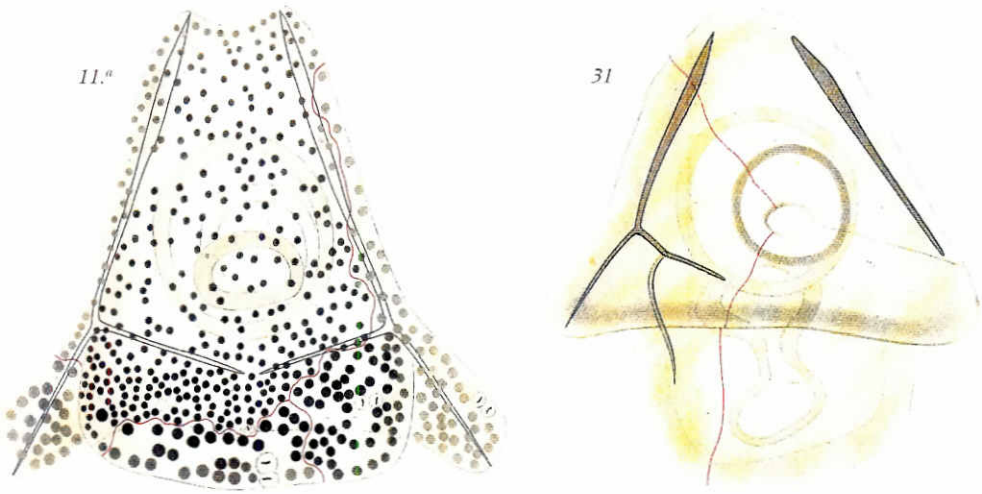


Fig. 5. *Paracentrotus larvae* developed from dispermic eggs. *The eggs cleaved simultaneously into three primary blastomeres (dispermic simultandreier).* **(11a)** *Quite normal larva, front view. The lines indicate the borders of the three regions, each descended from one primary blastomere. Because the triploid chromosomes were unequally distributed at the tripolar mitosis of the egg-cell, the nuclei of the three regions differ in size. As Boveri observed, at the back of those embryos, the lines always converge at the blastopore.* **(31)** *Pluteus with a sectorial defect. The left skeleton is normal, the right one consists only of the body rod. From Boveri (1907).*

by the uptake of nutritious substances, and by cell proliferation.' This phrase expresses the most important presupposition of cell biology in the conceptual framework of Mendel's hypothetico-reductionistic experimental approach: monospermy and the equivalence of gametes (Friedrich Gärtner had already shown that hybrid offspring are uniform in reciprocal crosses, nowadays known as Mendel's 1st law). From his own results Mendel drew the following conclusion (1866, p.59): 'Because we do not observe any alterations in the habitus of the hybrid plant during its whole life span, we have to accept that the differing elements (the alleles) would not succeed to give up their association until the stage of gamete formation. During the formation of those cells all elements (genes) are involved in a completely independent and symmetrical order whereby the differering elements exclude each other.' This deduction clearly defines the stage at which the single-copy state of genes is achieved in the life cycle of diplonts. Although the vehicles of inheritance, the chromosomes, were yet undetected, Mendel's view anticipates the process of reduction of the diploid to the haploid state.

Thus we are confronted with the suprising fact that (most) zoologists were completely unaware of the pioneering results of botanists in cell biology, especially of the work focused on fertilization. It was O. Hertwig who once and for all established the nature of fertilization in animals by his demonstration that the second nucleus observed in the egg just after fertilization originated from the sperm. He further observed the fusion of male and female nucleus. In a seminal analysis of the fertilization process, van Beneden (1883) demonstrated that the male and female pronucleus in the zygote of Ascaris (*Parascaris equorum*), without prior fusion, contribute each two chromosomes to the first cleavage division (Fig. 2: 47). The leading cytologist at the time, Weismann, proposed that the chromatin was the hereditary substance. He further established the theory of the germline, and predicted some kind of reduction division prior to gamete formation.

In an attempt to definitively prove the exclusive role of the nucleus in the emergence of the phenotype, the then 26 years old Boveri (1889) tested a brilliant idea, based on an experiment done by O. u. R. Hertwig. They had observed that fragments of sea urchin eggs without a nucleus could be fertilized. Those haploid merogonic fragments were capable to undergo cleavage. Boveri repeated this experiment and succeeded in rearing those haploid embryos into normal (dwarf) plutei. Then he executed the famous merogonic-hybrid experiment, by supplying sperm from a different species which displays a distinct phenotypic marker, the shape of the larval skeleton, that is different from the species from which the egg-fragment was obtained. The apparently successful experiment was published under the selfexplanatory title 'A sexually conceived organism without maternal traits' (1889). E.B. Wilson (1918) considered it a key experiment, 'a germ from which spread new lines of growth'. (In retrospect, one may consider the import of a foreign genome into a cell as the first 'DNA transformation' experiment). At that time things had already taken a tragic turn. Boveri, who considered this his most important experiment, was unable to repeat it and finally became convinced that he had observed an artefact. True to his motto to describe 'not only what is new but what is true', the ailing Boveri instructed his wife to publish a retraction and explanation. He explained one source of error in the persistance of some maternal chromosomes in the egg fragment which must cooperate with the sperm-derived chromosomes to allow post-blastula development in the hybrid (1918).

As early as 1887 Boveri had concluded from his cytological analysis of fertilization in Ascaris that chromosomes are continuous organelles. Since the polar bodies persist during embryogenesis and the chromosome number can be computed, Boveri was able to correlate several numerical chromosome abnormalities, observed at later cleavage, with irregularities during egg maturation, i.e. meiotic nondisjunction (Fig. 4).

Boveri (1909) distinguished several types of chromosome grouping in metaphase plates at early cleavage. He observed these configurations to be always identical at telophase of sister cells. In the ensuing prophase he again observed mirror-image chromosome groupings in sister cells (Fig. 2: 76.,81.-83.). Furthermore, the several types of chromosome arrangement occurred in the same frequencies he had estimated from metaphase plates of the preceding cleavage mitosis. Obviously, the movement of chromosomes, in the course of random engagement of their kinetochores with astral Mt's, cause the variable grouping in metaphase plates (Fig. 2: 56., 57.). However,

chromosome displacement (or even disintegration followed by new formation) does not occur during interphase, in spite of chromatin de- and recondensation. Boveri summarized his findings: 'I regard the chromosomes as the most elementary organisms which carry on an independent existence within the cell'.

Continuity and individuality of chromosomes seem to us merely the two sides of the same coin. Not so at the time before 1900. Weismann (1892), in his chromosome theory of inheritance, considered each of the mitotic chromosomes to contain the entire germline genome. Such was the case in *Parascaris univalens* (Fig. 7). Because of their variable amount of germline-limited chromatin, the germline chromosomes of Ascaris are structurally highly polymorph. Therefore, Boveri, cytologist second to none, was unable to distinguish the homologues in *P. equorum*, either in mitosis or at the stage of meiotic synapsis which contains two tetrads. The existence of individual chromosomes, recognizable by size, was shown by T.H. Montgomery and W.S. Sutton in grasshopper spermatocytes. Significantly, they demonstrated that each two similar chromosomes always pair up at meiosis. However, constant size-differences did not eliminate the possibility that morphologically different chromosomes might nevertheless have similar genetic properties. Boveri (1902) excluded this possibility definitively by an ingenious analysis of dispermy in sea urchins. By means of this experiment of Nature he demonstrated that chromosomes cannot be developmentally equivalent. By a quantitative analysis he (1907) succeeded in estimating the number of 'genophores' that are essential to control ontogenesis. This number corresponds nicely with the haploid number of chromosomes all of which looked almost alike (see Fig. 3b).

Using high sperm concentrations, double fertilization of sea urchin eggs can be achieved at high frequency. Due to their tetrapolar MA's, dispermic eggs divide simultaneously into four blastomeres at the first cleavage step. Because of the high cleavage synchrony in single egg batches these 4-celled embryos, named dispermic simultanvierers, can be separated from normal (monospermic) 2-cell embryos after the first cleavage. Boveri recognized that the primary blastomeres of dispermic eggs could be used as test-tubes to study the developmental significance of chromosomes. From his Ascaris studies he knew that the chromosomes are randomly distributed to the partial spindles – up to six – in dispermic egg-cells. Consequently, three sets of chromosomes are unequally distributed to the four primary blastomeres (see Fig. 11 in K. Sander's contribution in this volume). If different chromosomes bear different genetic properties, then, as a consequence of their random distribution, defects should arise during development. Boveri first demonstrated that all four blastomeres, isolated from a single normal 4-cell stage, are capable to develop into dwarf plutei. When he repeated this exeriment with dispermic simultanvierers the four sister-cells, cultured in isolation, displayed quite different developmental potentials. He wrote (1904a, p.47): 'one quarter; for example, may decay into isolated cells, one may remain in the blastula stage, a third may gastulate before ceasing development at this stage, whereas the fourth may form spicules... and thus beginning its transformation into a pluteus' (see Fig. 11 in Sander's contribution in this issue). If the cause of developmental failure of blastomeres isolated from simultanvierers were the result from chromosome shortcoming, then the non-separated simultanvierers would be expected to develop sectorial defects. This was exactly what Boveri observed (Fig. 5). This restricted and different developmental potential did not depend on nuclear size, i.e. on chromosome number. From all these data Boveri (1902, p.75) concluded 'not a certain number but a certain combination of chromosomes is required for normal development, therefore individual chromosomes must possess different qualities.'

At this level of insight Boveri (1907) went on to estimate the exact number of different chromosomes, which is necessary to guide normal embryogenesis, from the frequency of healthy larvae developing from aneuploid cells. This type of quantitative analysis will work only under two preconditions: (i) each dispermic egg or blastomere after the first division in principle is capable to develop regulatively into a normal larva (this is by no means trivial. It does not hold for the primary blastomeres of dipermic eggs in Ascaris. See chapter 'Ascaris studies'), (ii) each primary blastomere is able to cooperate in that process if its chromosome complement, irrespective of chromosome number, contains at least one copy of all the different chromosomes necessary in embryogenesis. Together with his wife, Boveri isolated more than 1.500 dispermic simultanvierers, and only one completed embryogenesis. As suggested from the high chromosome number (n= 18), the high frequency of pathological development pointed to a large number of nonequivalent essential genophores. As a consequence of the relation between the number of different genophores (n) and the probability (p) to obtain healthy larvae from triploid dispermic simultanvierers, given by the equation $p(n) = 0.528^n$, Boveri's result pointed to the presence of more than nine different chromosomes as crucial supply for normal development (see Moritz, 1993, for details). In other words, his finding ruled out the possibility of a diploid set in gametes. One can easily deduce that healthy cases of development will be more frequent after a tripolar than after tetrapolar cleavage division. Such dispermic simultandreiers arise frequently when eggs are shaken. Boveri isolated 719 simultandreiers and got about 11 per cent healthy larvae (Fig. 5). As a consequence of the equation $n= \ln f \times (\ln 0.889)^{-1}$, which defines the relation between the relative frequency of healthy larvae (f) and the number of different essential genophores (n), Boveri's result of f= 0.11 amounts to n= 18 as the number of different chromosomes. To confirm this result, Boveri investigated the developmental potential of primary blastomeres isolated from dispermic simultandreiers. There are four types of non-segregated simultandreiers (D_0, D_1, D_2, D_3) consisting of $3(D_0)$, $2(D_1)$, $1(D_2)$, or $0(D_3)$ aneuploid blastomeres, respectively, each of which contains a full haploid set of 18 chromosomes. These types are expected to occur at the following probabilities (p): $p(D_0)= 0.12$, $p(D_1)= 0.39$, $p(D_2)= 0.38$, and $p(D_3)= 0,11$. Accordingly the frequency of healthy primary blastomeres, reared in isolation, amounts to $p(D_0) + 2/3\ p(D_1) + 1/3\ p(D_2)= 0.51$. Boveri isolated 102 blastomeres from 34 simultandreiers and obtained 44 quite normal gastrulae. This result and, furthermore, the distribution of the embryos with reference to the four types of dispermic simultandreiers were in fairly good agreement with the expected relative frequencies. To summarize: Boveri demonstrated that the number of different genophores necessary to guide embryogenesis is equal to the haploid chromosome number. In other words, the genome of the sea urchin does not contain extra elements nor chromosomes of submicro-

scopical size. Boveri's quantitative analysis has never been adequately appreciated by his peers up to this day (see Moritz, 1993; for description of the 'random machine' which Boveri used to estimate the respective probabilities see Sander, 1994). For developmental biology, two further deductions are important. (1) Nonbalanced chromosome complements, i.e. multiple copies, apparently do not destroy the potential of normal development in most cases. (2) The early atypical cleavage does not disturb later conditional programming. In the mosaic embryo, cells may differ in size and number according to the constant species-specific nuclear-cytoplasmic ratio. Nevertheless, they cooperate in the formation of normal organs. Especially, the simultandreier is capable to establish bilateral symmetry (Fig. 5).

From all his seminal cytological and cytogenetic data which include the analysis of meiosis in several organisms, Boveri concluded that the MA of the reduction division is unable to distinguish homologous chromosomes with respect to their parental origin. Therefore, this division may generate multiple combinations of maternal and paternal chromosomes (2^n) to establish new haploid sets in gametes (1904a, p. 88). Besides such interchromosomal recombination, he proposed intrachromosomal recombination at the meiotic stage of synapsis. As a complementary approach to his strategy to estimate the number of essential genophores by a quantitative analysis of somatic genetic mosaics, he proposed to execute crossing-experiments to reveal genetic linkage and to establish linkage groups. This was done in the following decade by T.H. Morgan and his group in the famous 'fly-room' at Columbia University. In his impressive synopsis 'Results on the constitution of the chromatic substance in the cell nucleus' combining the facts about chromosomes with the Mendelian law of heredity, Boveri characterized this synthesis as follows (1904a, p.117): 'We see here that two areas of study which developed quite independently of each other have yielded results which are as harmonious as if one had been derived theoretically from the other.' Today, with hindsight, we can fully appreciate the conceptual progress achieved by Boveri – according to E.B. Wilson (1918, p.74) Boveri's 'crowning achievement, whether in respect to excellence of method or importance of result'. However, at his time we must remember that Boveri's ideas, which we now call the Sutton-Boveri Theory of Inheritance (see also Sutton, 1903), were met with strong skepticism. One reason was that at first there seemed to be no definitive evidence connecting any specific heritable trait with one certain chromosome. However, sex determination was the character which supplied such evidence to Boveri (1892). Later, he studied the transmission of sex chromosomes in both sexes of Ascaris and *Angiostomum (Rhabditis) nigrovenosum*. The latter species is characterized by an obligatory alteration of one generation of a free-living dioecious form followed by a parasitic hermaphroditic form. Besides Waldemar Schleip, Boveri clarified this alteration as a consequence of an atypical behavior of X-chromosomes in spermatogenesis which results in the formation of only one functional sperm type (X) in the dioecious form but two types (X and nullo X) in the hermaphrodite.

One final note on the impact on the academically and intellectually pleasing tour de force that culminated in the Chromosome Theory of Inheritance: It concerns the potential role of chromosome disorders in human disease. Since he had observed certain abnormalities concerning cell proliferation and cell adhesion in the early cleavage of aneuploid dispermic eggs in sea urchins, Boveri postulated that certain chromosomes may control several basic cellular traits. An offshoot of this finding is his small tome 'On the origin of malignant tumours' (1914), where he proposed that tumors may result from derangements of certain chromosomes which are involved in cell-cycle control. This little book, translated by his wife in 1929, is the only original work of Boveri available in English. It provides the first clear vision of cancer as a cell-heritable disease. Specifically, the cancerous state after removal of a chromosome or a fraction thereof somehow preceded the discovery of tumor suppressor genes.

Boveri's theory of regional and cellular specification in early embryos

Although Boveri is mainly remembered in connection with the Chromosome Theory of Inheritance and the dominant role of the nucleus in cellular activities, work performed over a quarter century of cell studies (1885-1909), he has also been a major personality in the emerging field of experimental embryology. He had the good fortune to be working on the sea urchin and Ascaris, two embryos at opposite ends of a scale of embryonic systems known as regulative and mosaic eggs, respectively. This, together with his exceptional experimental talent, his precise logic, and a gift for imaginative associations, enabled him to discover universal principles of development. Surprisingly, as we shall see, sea urchin development revealed traits of preformation and that of Ascaris epigenesis and a common principle, still not fully understood and perhaps best described as *polarization*. He observed little evidence for cell mixing among the off-spring of founder cells, which made it possible to trace cell-histories and to construct fate maps. Most of his work on sea urchins was done at the Zoological Station at Naples, the early center of Developmental Biology and gathering place of congenial colleagues from all over the world.

Sea urchin studies

As early as 1889 Boveri had shown that small egg fragments are able to develop into normal dwarf larvae. Hans Driesch demonstrated for the first time that 1/2- and even 1/4-blastomeres (i.e. after the first or second cleavage cycle, respectively), when cultured in isolation, develop into complete larvae. From this remarkable phenomen of regulation Driesch reasoned that the sea urchin embryo behaved unlike any known machine, because of its capacity of self-repair in connection with cellular reproduction. He described the embryo as a 'harmonious equipotential system' that reveals qualities restricted to living systems. Driesch thought he discovered evidence for a vital force which he named 'entelechy'. He drifted off into vitalism and wrote treatises like 'Die Philosophie des Organischen'. Boveri maintained that Driesch may have done a bad experiment as he rushed to the conclusion of the 'harmonious equipotential system' from his experiments with 4-cell stages. Boveri extended the experiment to the 8-cell stage and found that isolated blastomeres and partial embryos were no longer omnipotent. Only about half of them regulated into larvae while the other half stopped developing at the blastula stage. As is typical for Boveri's style of research, his analysis was based on comprehensive case studies, where the developmental history was

meticulously chronicled by live observation, drawings, and final preservation and staining. But in these early isolation experiments it was not certain which blastomeres were derived from which region of the egg-cell. Nor was it possible to trace their relations to those cells of the blastula, the vegetal plate, where the first cellular specifications take place and gastrulation movements begin. Boveri (1901b) solved this problem which also allowed him to draw a fate map of the sea urchin embryo by discovering two reliable markers of orientation (Fig. 6). By placing eggs of *Paracentrotus lividus* in india ink he noted a narrow channel in the jelly coat which surrounds the egg. The funnel probably marks the site of attachment of the oocyte in the gonade and is the definitive location of polar-body formation. Consequently, this point precisely defines the animal pole. Boveri observed a pigment ring covering a wide segment of the vegetal half of the egg-cortex but excluding the pole regions. With these two distinct landmarks Boveri could trace the events of normal development. He proved that the blastopore arises near the vegetal pole and drew a fate map which contained the ectoblastema (the colorless animal cap), the endoblastema (the colored region) and the prospective micromeres (source of primary mesenchyme) nearest to the colorless vegetal pole. An important observation demonstrated that the pigment ring was not a permanent fixture but arises from cytoplasmic rearrangements which are initiated during egg maturation and concentrate the globally distributed pigment granules in the subequatorial ring (Fig. 6). Here is a visible indicator of cell polarization, i.e. cytoplasmic anisotropy, confirming his conclusions of his earlier egg fragmentation experiments with unpigmented material.

Even a more dramatic diversification of up to five different colored regions was observed by two influential embryologists in eggs of two different animal phyla; *Styela*, a tunicate, studied by Edwin G. Conklin, and *Dentalium*, a mollusc, studied by E.B. Wilson. The remarkable similarity of orientation of the colored cytoplasm relative to presumed organ-forming regions in the respective eggs, together with experimental results that were consistent with mosaic development, provided most powerful arguments for the theory of 'prelocalization'. However, Boveri's findings were made on a paradigm of 'regulative' development. Does that mean that there are local determinants in the sea urchin egg which are responsible for the anisotropy demonstrated in the series of isolation experiments? In contrast to numerous classical and recent molecular studies, all postulating determinants in the micromeres, Boveri's answer was: no. Using appropiate *Paracentrotus* 'ring-eggs' he was able to state unequivocally from which region of the egg isolated fragments or partial embryos were derived. This enabled him to do the following experiments.

He succeeded in splitting the egg along its animal-vegetal axis, just as it happens in the first cleavage step (1901a). Then he fertilized those fragments and obtained normal dwarf larvae. In the crucial experiment he split the egg at the equator, as it normally happens at the third cleavage step (Fig. 6, 18.). After fertilization he obtained a normal larva from the vegetal half but only a blastula from the animal half. Clearly, the mature egg is anisotropic along its animal-vegetal axis, and the anisotropy is maintained during the first two meridional cleavage-divisions. In other words, whatever Driesch considered evidence for 'entelechy' became amenable to experimental analysis. By several strategies Boveri produced embryos that did not cleave off micromeres. Nevertheless, those embryos produced primary mesenchyme, and hence formed a normal skeleton. Elongation of the egg-cell along the equatorial region, by compressing the embryo, resulted in duplication or even triplication of the archenteron. This important finding appears to foreshadow the amphibian organizer studies – considered by some the most important result of the era of experimental embryology – done by Hans Spemann, Boveri's most famous student. By precisely following the cleavage pattern and the developmental potential of egg fragments and partial embryos derived from 'ring-eggs', Boveri observed a consistent correlation between developmental success and the distribution of the yellow cytoplasm. He drew the general conclusion that gastrulation begins at the relative highest level of the colored region, and that this 'privileged region', characterized by some invisible material at its highest concentration, acts as an 'activation center' for the first embryonic cell-specification. In cases of duplicated archenterons, apparently the organizing center had been split into two in the flattened embryo, and differentitation apparently occurs by interaction with the center, i.e. via short-range induction. Boveri (1901a, p.167) suggested that 'any region of the blastula (with the exception of the animal hemisphere) is able to gastrulate or to form mesenchyme, and that the localization of these events in the intact embryo to a single site is determined by the ease with which these processes can be initiated in that region rather than at any other place'. He finally modified Driesch's famous aphorism 'Each part, if need be, can do everything', to (1901a, p.169) 'Each part can do it, provided the one which could do it better is no longer present. The one that can do best, however, is probably the relatively most vegetative.'

Considering Driesch's machine dilemma, Boveri reasoned (1902, p.85) 'Those primitive differences in the ooplasm as indicated by the visible layering, which are transmitted to the cleavage stage embryo in unchanging topological relations, may influence differentially the primarily omnipotent nuclei, thereby leading to activation or repression of certain nuclear qualities, as can directly be seen in the cleaving Ascaris embryo. These nuclear differentiations may confer specific developmental potential to the cytoplasm which initially displays only a graded property.' Needless to say that at that time nothing was known about the molecular nature of genes, their coding function and selective expression as a result of specific processes, now named transactivation. Nevertheless, a great idea was born to explain ontogenetic cell-specification. Moreover, it is exemplified by a developmentally regulated genome-rearrangement, namely chromatin diminution in Ascaris (see Fig. 7). Boveri's vision of 1902 of a transient quantitative state of the cytoplasm as a primary cause in the process of cell commitment, although little remembered, took the place previously occupied by the concept of 'organ-forming stuff '. His conclusions on the dynamic architecture of the sea urchin egg and embryo exclude any requirement for local factors which become segregated into and, thereby, specify the micromeres, as is still postulated in current research and reviews of the sea urchin system. In embryos which fail to form micromeres, the presence of an organizing center near the vegetal pole of the egg-cell seems sufficient to induce directly the founder cells of the primary mesenchyme. In full agreement with Driesch, Boveri reasoned that the fate of a

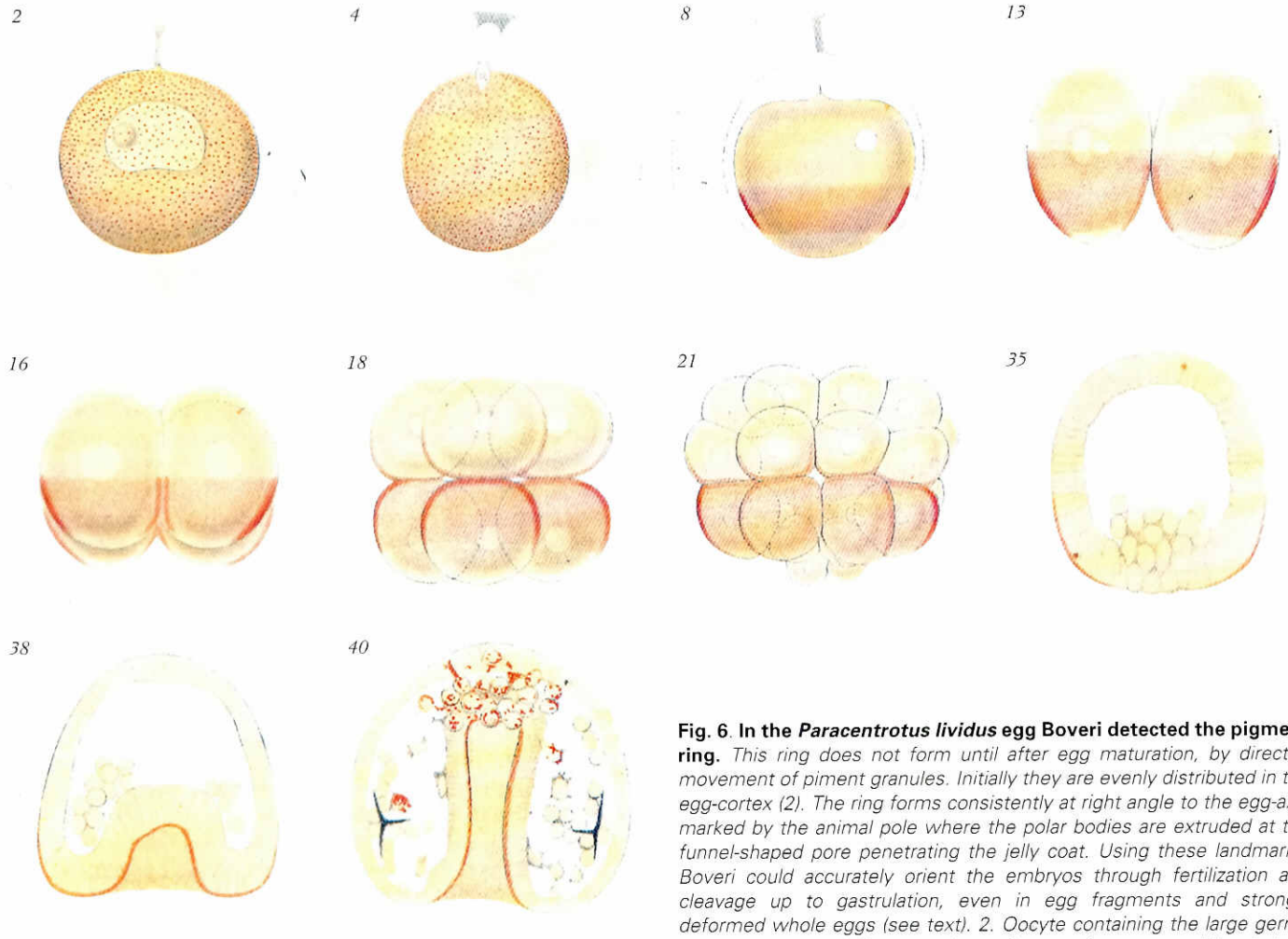


Fig. 6. In the *Paracentrotus lividus* egg Boveri detected the pigment ring. *This ring does not form until after egg maturation, by directed movement of piment granules. Initially they are evenly distributed in the egg-cortex (2). The ring forms consistently at right angle to the egg-axis marked by the animal pole where the polar bodies are extruded at the funnel-shaped pore penetrating the jelly coat. Using these landmarks, Boveri could accurately orient the embryos through fertilization and cleavage up to gastrulation, even in egg fragments and strongly deformed whole eggs (see text). 2. Oocyte containing the large germinal vesicle, 4. polar body formation, 8. the mature egg-cell after pigment ring formation, 13. 2-cell stage, 16. 4-cell stage after two meridional cleavages, 18. 8-cell stage after equatorial division, 21. 24-cell stage, four micromeres were formed by asymmetrical cleavage of the vegetal blastomeres, 35. primary mesenchyme formation, 38. archenteron invagination, 39. secondary mesenchymal cells disperse into the blastocoel from the top of the archenteron. From Boveri (1901b).*

nucleus, or a blastomere that is carved out of an egg or an egg fragment during cleavage is dependent on its relative position in the (whole or partial) embryo. This notion can serve as the foundation of the 'positional information concept'.

Comparing the developmental potential of merogonic hybrids, true species hybrids, and dispermic (homospermic) eggs, Boveri (1918) was able to contribute further insights into the interplay between chromosomes and cytoplasmic factors. From the diverse defects seen in the latter experimental egg-type he had concluded that each chromosome of the haploid set is needed in embryogenesis. On the one hand, merogonic hybrids stopped almost always development at the late blastula stage without further differentiation. On the other hand, species hybrids – i.e. using the same pair of species to produce hybrids and merogons – may develop into larvae which often displayed paternal traits. From these results Boveri concluded that development proceeds essentially in two steps. The second, beginning in the blastula stage requires the control by the chromosomes, the first may not. The first phase, which includes cleavage pattern and cleavage rhythm typical of the mother, the establishment of body axes, regionalization and shape of the early embryo, was described as generating the general 'promorphological form'. To this extent phase one was characteristic of 'preformation' and has been compared to a 'rough sketch of a picture where the details are filled in by nuclear actions' (1907, p. 254) – or more precisely by cytoplasmic-nuclear interactions – during phase two development. Again, Boveri went one step further backwards and argued that the conditions for phase one development were set up under the influence of the maternal genome during oogenesis. In todays parlance, Boveri distinguished between the two consecutive phases of maternal and zygotic control of embryonic patterning. From the observation that the nuclei in heterosperm merogons, albeit capable to undergo numerous normal cleavage divisions, were unable to cooperate with the ooplasm to manage post-blastula morphogenesis, Boveri (1918, p. 411) stated that 'the role of the cytoplasm is much more specific than believed until now'. The occurrence of paternal traits in true species hybrids, on the other hand, points to a more unspecific

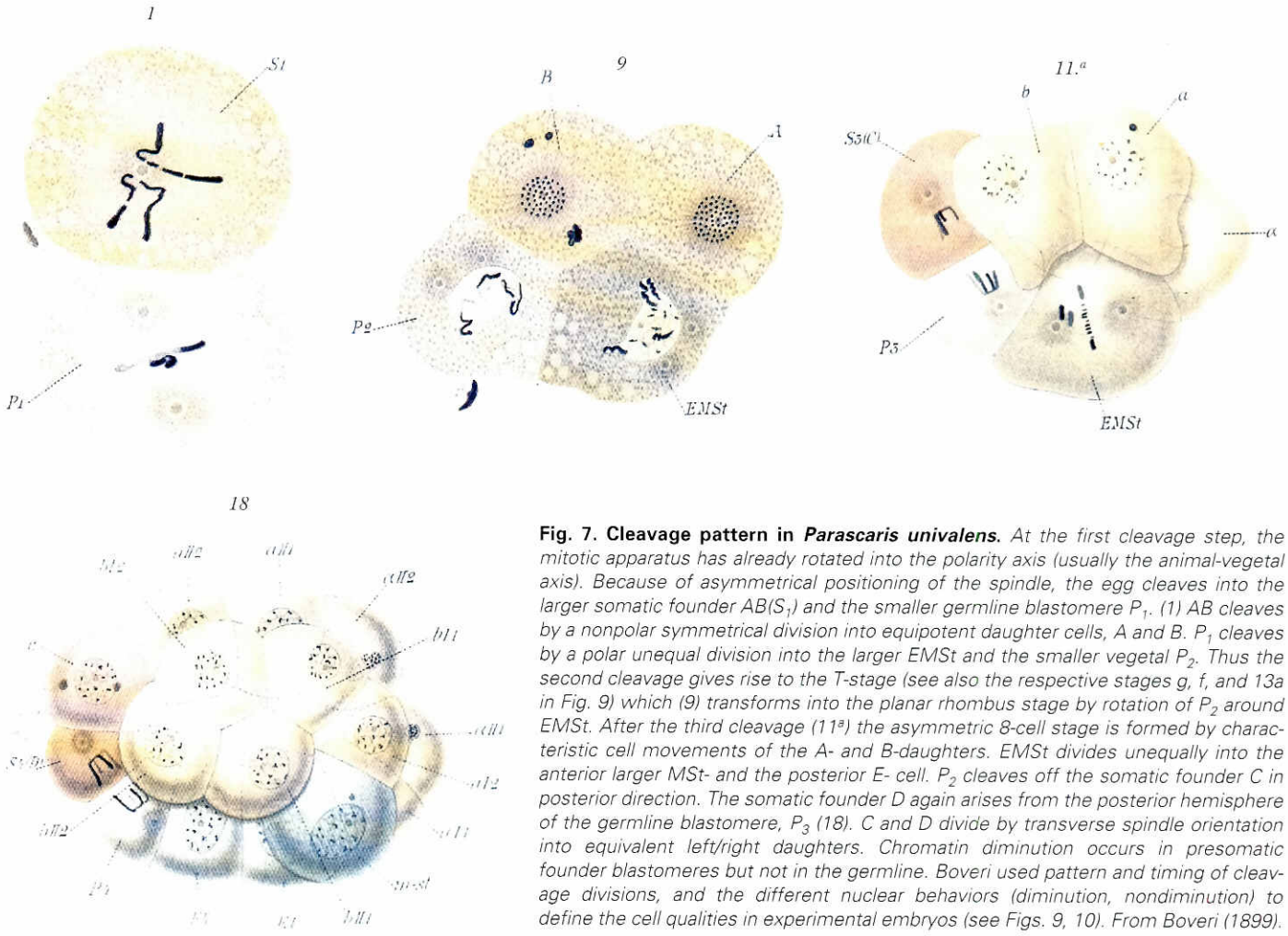


Fig. 7. Cleavage pattern in *Parascaris univalens*. *At the first cleavage step, the mitotic apparatus has already rotated into the polarity axis (usually the animal-vegetal axis). Because of asymmetrical positioning of the spindle, the egg cleaves into the larger somatic founder AB(S_1) and the smaller germline blastomere P_1. (1) AB cleaves by a nonpolar symmetrical division into equipotent daughter cells, A and B. P_1 cleaves by a polar unequal division into the larger EMSt and the smaller vegetal P_2. Thus the second cleavage gives rise to the T-stage (see also the respective stages g, f, and 13a in Fig. 9) which (9) transforms into the planar rhombus stage by rotation of P_2 around EMSt. After the third cleavage (11[a]) the asymmetric 8-cell stage is formed by characteristic cell movements of the A- and B-daughters. EMSt divides unequally into the anterior larger MSt- and the posterior E- cell. P_2 cleaves off the somatic founder C in posterior direction. The somatic founder D again arises from the posterior hemisphere of the germline blastomere, P_3 (18). C and D divide by transverse spindle orientation into equivalent left/right daughters. Chromatin diminution occurs in presomatic founder blastomeres but not in the germline. Boveri used pattern and timing of cleavage divisions, and the different nuclear behaviors (diminution, nondiminution) to define the cell qualities in experimental embryos (see Figs. 9, 10). From Boveri (1899).*

activation of those genes which are involved in terminal cytodifferentiation.

In summary, Boveri's thorough analysis of sea urchin development led to insights into the dynamic organziation of the egg, the interaction between cytoplasm and the nucleus, specifically the emergence of a cytoplasmic organizing center, and local cell-to-cell interaction, that both predate and challenge current wisdom.

As a paradigm of 'regulation', the sea urchin egg is less of the 'harmonious equipotential system' as envisioned by Driesch. However, no local determinants need to be postulated to explain formation of the embryonic pattern. This apparent paradox has been unraveled by Boveri in his far-reaching concept of segregative cell-division. This concept explains the harmony of topogenesis and typogenesis of blastomeres. Typogenesis depends on a certain cytoplasmic milieu which acts on the nucleus, i.e. via transaction on chromatin; topogenesis depends on a certain dynamic cytoplasmic architecture which acts on the MA, i.e. on the positioning of the centrosome, the other permanent organelle. Consequently, the dynamic processes which exert control over the centrosome in a mother cell must also provide the preconditions for the generation of a certain cytoplasmic milieu which differs in daughter cells at segregative cleavage divisions. We shall learn more about the dynamics of the cytoplasmic organization – its recurrent architectural rearrangement and its indirect influence on the nuclear activity – as an epigenetic process, from Boveri's analysis of the cleavage potential of the Ascaris egg, a paradigm of mosaic development.

Ascaris studies

Boveri's first original contribution to the Ascaris system was his discovery of chromatin diminution (1887b). In certain blastomeres he observed the disintegration of the large chromosomes into numerous small chromosomes and large blocks of heterochromatin (these plurivalent compound chromosomes consisting of the somatic genome and germline limited chromatin, are characteristic for Ascaris and some other animals' germline). The latter fail to move at anaphase and are thus eliminated from the daughter nuclei. Moreover, he noted that diminution was restricted to presomatic blastomeres. He went on, over a period of 12 years, to precisely determine the invariant cell-genealogy of the embryo up to the 176-cell stage (Fig. 7). From his intimate knowledge of each blastomere's normal fate, documented by an exquisite set of colored lithographs, he constructed a lineage tree (Fig. 8). This tree indicated the germline maintaining the zygotic karyotype, and the descendants of four somatic founder cells which arise by

four successive asymmetric cleavage divisions, each contributing a predictable number of cells and cell-tpyes to the larvae. This work (1899), dedicated to his teacher Carl v. Kupffer, was judged by E.B. Wilson (1918) as a classic publication in developmental biology, unsurpassed by any other study on cell genealogy. Only recently has the lineage-tree of *Caenorhabditis elegans* been fully unraveled. Because its development is very similar to that of Ascaris, Boveri's conclusions can be related to a developmental system that is currently under intense analysis by modern tools including molecular genetics.

Before we turn to Boveri's concept of autonomous cell-fate specification in Ascaris, an alternative view needs to be discussed that seems to be consistent with Weismann's original chromosome theory. According to a prediction from that chromosome theory, ontogenetic cell differentiation would be a result of genetically unequal chromosome segregation. In this view the autonomous behavior of the chromosomes during 'differential mitosis' explained why cells become different. At first sight, chromatin diminution could be the result of such segregative mitoses. As suggested by Otto zur Strassen, as late as 1957, the chromosomes of the zygote and their germline daughter-cells would consist of one chromatid programmed to undergo diminution. After each division, the cell daughter inheriting the chromosomes destined to diminute became a somatic cell. The unchanging sister chromatids, by their segregation along the other cell-line, would cause the germline lineage to end up in the germ cells.

Boveri definitively ruled out this interpretation by two experiments. In the first he made again use of an experiment of Nature, dispermy (1904b). Dispermic egg-cells of *Parascaris equorum (Ascaris meg. bivalens)* contain three nuclei each containing two chromosomes. Consequently, according to the postulate of autonomous chromosome behavior, six chromosomes should preserve their germline state, not only at the earliest stages but also in the germline of later embryos. Because of the random distribution of the chromosomes at the first tetrapolar mitosis of dispermic eggs, both types of chromosomes should be incorporated into the same daughter cell. In other words, diminution and nondiminution should occur in a single mitotic division. These predictions are not fulfilled. In each case all chromosomes, irrespective of number, behave the same in a certain cell: either all or none undergo diminution (Fig. 9).

The second experiment involved centrifugation of a normal egg during the first cleavage cycle (1910a). If the centrifugal force acts along the animal-vegetal axis of the egg, the spindle is held along its equatorial plane (Fig. 4: 14.), and the cell divides along its animal-vegetal axis. These are the famous 'ball-embryos', since at the animal pole where the most dense particles gathered, a little exovate (ball) is extruded (Fig. 10a). In contrast to the normal first cleavage which separates the animal and the vegetal hemispheres and gives rise to AB- und P_1 -blastomeres, the 'ball-egg' cleaves meridionally and symmetrically. This equal division leads to identical behavior of all chromosomes (no chromosome undergoes diminution). Again, this does not support the hypothesis of autonomous differential chromosome segregation to explain blastomere commitment in the mosaic-type of development. From these observations Boveri concluded that the mutually exclusive chromosome behaviors, diminution or nondiminution, must be under the control of the cytoplasm: 'the only possibility remaining is that the composition of the cytoplasm determines the fate of the individual chromosome into one or the other direction' (1910b, p.179). Recently, Boveri's conclusion has been confirmed directly. Subsequent to induced mitotic nondisjunction at the first cleavage mitosis, the nonsegregated sister-chromatids behave identically whether they are incorporated into P_1 or AB. They undergo chromatin diminution in presomatic blastomeres, but not in germline blastomeres (Seidl *et al.*, 1988).

Boveri's interpretation first published in a short communication entitled 'Protoplasmic differentiation induces nuclear differentiation' (1904b), became widely accepted, although with tragic consequences for Boveri's paradigm of autonomous cell-fate specification. In fact, Boveri's first rank discovery, according to E.B. Wilson (1918), became a crucial argument for the existence of prelocalized cytoplasmic factors. These are believed to act as 'determinants' in the process of cell-type specification in the early embryo. The theory of prelocalization is closely linked with the concept of mosaic-type of development and seems well supported by classical experimental results with *Styela* and *Dentalium*, alluded to in the sea urchin section. A recent comprehensive review of the classical literature, mostly through the influential text of E.B.Wilson (1925), comes to the conclusion that prelocalization of determinants in eggs is 'a demonstrable reality, and not an illusion' (Davidson 1986, p. 411). Together with the current 'Genetic Theory of Development' based on the concept of 'differential gene expression', local factors, or prefactors, make good candidates for specific trans-acting factors. They would control the activity of 'smart genes', the products of which, in turn, cause regional cell-fate specification in the early embryo. At first sight, the celebrated cases of local mRNA in *Drosophila* and *Xenopus* eggs seem to prove the current dogma. But these cases are the result of events more downstream from the still mysterious process of 'localization'. Significantly, similar regulative molecules in the cytoplasm or local membrane domains have remained elusive in the classical cases of *Styela* or *Dentalium*.

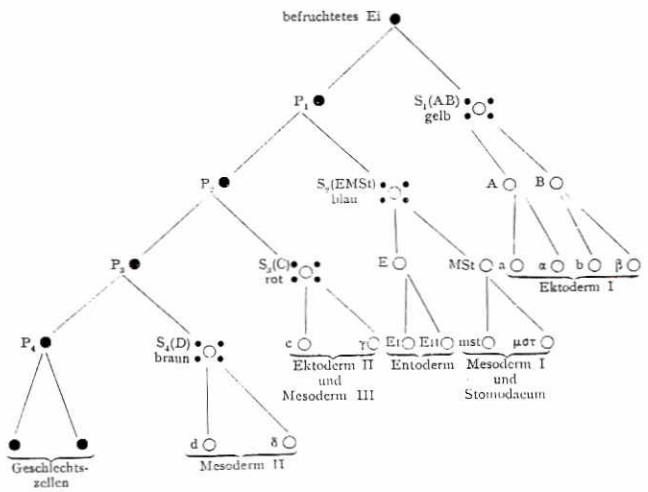


Fig. 8. Early lineage tree of *Parascaris spec.* Segregation of germline and presomatic cells. *As the prospective fates indicate only the intestine and germ cells are clonally derived. Mesodermal, ectodermal, and neural tissues are of polyclonal origin. Diminution is indicated by dots. In Figures 7, 9, and 10 color codes of the presomatic blastomeres and their descendents are the same as indicated here. From Boveri (1910b).*

Fig. 9. ***Parascaris*** **dispermic eggs.** *These eggs divide into 4 blastomeres at the first cleavage division (dispermic simultanvierer). Three types have been observed: type I: 3AB's + 1P$_1$; type II: 2AB's + 2P$_1$'s; type III:1AB + 3P$_1$'s.* **(a)** *Type I: 2a, g live observations, 2m whole mount preparation. P$_2$ cleaved into the larger posterior C and the smaller sister P$_3$.* **(b)** *Type II: 6a, f live observations, 7g whole mount preparation. The germline blastomeres, P$_3$'s, contain 6 chromsomes in total. Hence 6 chromsomes were distributed to the AB's. P. equorum (3n = 6).* **(c)** *Type III: 11, 13a live observations. In 13d (consisting of 13 cells) the 3 germline blastomeres contain 1+2+2= 5 chromosomes. Hence one chromatid was distributed to the single AB cell. P. univalens (3n= 3). The C-cells would have undergone diminution at the next cleavage. Compare with the 'ball-embryo' in Figure 10. From Boveri (1910b).*

Boveri's arguments against local blastomere-specifying factors are mainly based on the outcome of dispermy and centrifugation experiments. One might reason that, if the Ascaris egg is a structural mosaic of informational molecules, the four primary blastomeres which arise simultaneously from a dispermic egg might display four different fates – if any – according to those of the normal 4-cell stage (A, B, EMSt, P$_2$). As Boveri unequivocally demonstrated, this is never the case (1910b). Instead the four blastomeres constantly display only the two alternate states at the 2-cell stage: either AB or P$_1$ (Fig. 9). Extensive case studies over more than ten years revealed three types of embryos; those with 1, 2, or 3 P$_1$-cells and 3, 2, or 1 AB-cells, respectively. As the position of the second polar body and the partitioning of the deutoplasmic material to the four primary cells indicate, the dispermic egg-cell can divide at *any* plane with respect to the animal-vegetal axis, and it always generates AB- and P$_1$-cells. This conclusion has important implications, because the often observed invariant cleavage-pattern of the paradigm of mosaic development (like spiral cleavage) is commonly believed to guarantee that blastomeres inherit the putative determinants, assumed to be localized at certain cytoplasmic domains. According to this concept, one is forced to ask

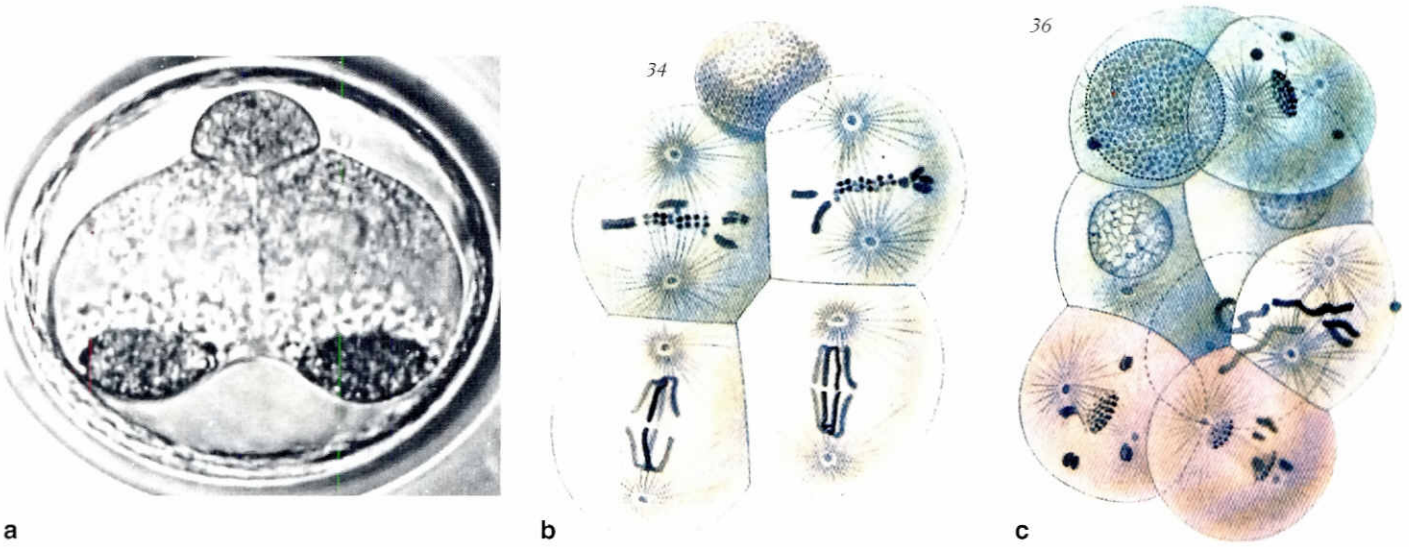


Fig. 10. Early cleavage of a 'ball-embryo'. (a) *Nonpolar symmetric cleavage (division parallel to the polarity axis) of P_0 zygote, achieved by centrifugal force (1800 g; microphotograph from Moritz 1967), results in lineage duplication. Boveri demonstrated that both daughter cells adopt normal P_1 fate. Each cell divides asymmetrically into a larger presomatic cell and a smaller vegetal germline cell. This 4-celled embryo looks like a normal 4-cell rhombus stage (Fig. 6: 9.), and like a type II dispermic simultanvierer; but it is neither: the normal embryo (after two cleavage cycles) consists of four different blastomeres, A, B, EMSt, and P_2; the type II simultanvierer (after the first cleavage cycle) is a complete twin: 2 AB's, 2 P_1's (Fig. 9: 6, Fig. 11d); but the 'ball-embryo' is a partial posterior twin.* **(b)** *The EMSt quality of the animal cells becomes clear from their polar spindle orientation and their diminution (34). The germline sister cells (P_2's) cleave off presomatic cells from the original vegetative pole (red colored cells).* **(c)** *Both these cells (C's) undergo diminution mitosis with paratangential spindle orientation (36). Each P-cell (P_3) produces one last presomatic cell (D), and each germline daughter (P_4) through a last symmetrical cleavage the two progenitors of germ cells (see the lineage tree in Fig. 7). These results led Boveri to abandon the concept of specific determinants and to replace it with cell-cycle dependent, graded quantitative changes of the cytoplasmic milieu as a prerequisite for cell-fate specification. From Boveri (1910b).*

what 'Deus ex machina' governs the species-typic cleavage pattern to manage the sorting out of informational molecules (perhaps Driesch's 'entelechy'?). Boveri concluded that the alternative fate of blastomeres is established by a mechanism of bifurcation which is intrinsic to the 'segregative cleavage step'. Consequently, since any cleavage angle seems to generate just two cell states, there is no need to postulate local cytoplasmic determinants.

As discussed above, by applying centrifugal force during the first cleavage cycle, the zygote may divide by a meridional (symmetrical) division. Closer analysis of the 'ball-embryo' proved that the two blastomeres display identical quality (1910b). However, they do not conserve P_0 -quality. They both adopt daughter-cell fate and become P_1 -cells (Fig. 10). In other words, the symmetrical division of P_0 resulted in duplication of the P lineage at the cost of lineage segregation into AB and P_1 . In the following asymmetrical cleavage-division of 'ball-embryos', daughter cells arise next to the 'ball' which incorporate the original animal cytoplasmic domain of the zygote. But both these cells never restore AB-quality. Instead, both blastomeres divide into a presomatic EMSt- and a P_2-cell, just as P_1 does in the undisturbed embryo. The indicated cell qualities become unequivocally clear at the third cleavage step: As is characteristic of normal cleavage, each EMSt undergoes diminution and divides unequally into a larger anterior MSt and a smaller central E cell (Fig. 10b). After reversal of cell polarity, each of both P_2's cleaves into a smaller P_3 and a larger C. The latter arise from the original vegetal pole of the egg-cell. The presomatic character of both C-cells is indicated by chromatin diminution and transverse spindle orientation at the fourth cleavage step which gives rise to equal-sized daughters (Fig. 10c). Each germline blastomere cleaves off a final larger presomatic blastomere from the posterior hemisphere, just as P_3 of the normal embryo divides asymmetrically into P_4 and the presomatic founder cell D. In summary, both primary blastomeres of the 'ball-embryo' execute the same series of asymmetrical cleavages, as P_1 does in the undisturbed embryo. Both nondiminuted cells, after the fourth cleavage, divide by an equal division into daughter cells which cease further proliferation, corresponding to the behavior of normal P_4-daugther cells, the progenitors of the germ cells. Hence, the 'ball-embryo' is a partial twin. Boveri further observed 'ball-embryos' without any ball-formation at the animal pole. On the other hand, after centrifugation, 2-celled embryos occurred that had extruded a ball from the animal region consisting of the typical dense granules. Nevertheless, their blastomeres displayed normal AB- and P_1-qualities. Hence, the extrusion of an animal cytoplasmic portion is neither necessary nor sufficient to generate this special partial duplication. Boveri discovered that, instead of stratification of a cell-type (AB or P_1) determining factor by centrifugation, the blockade of a 90° rotation of the MA (from the equatorial plane into the axis of polarity) explains the symmetrical division of the zygote P_0 into two equivalent cells. Specifically, he noted, after stopping centrifugation at metaphase, that – despite displacement of the putative factors – the MA actively turned into the animal-vegetal axis and always generated asymmetric cleavage into AB- and P_1-cells. Moreover,

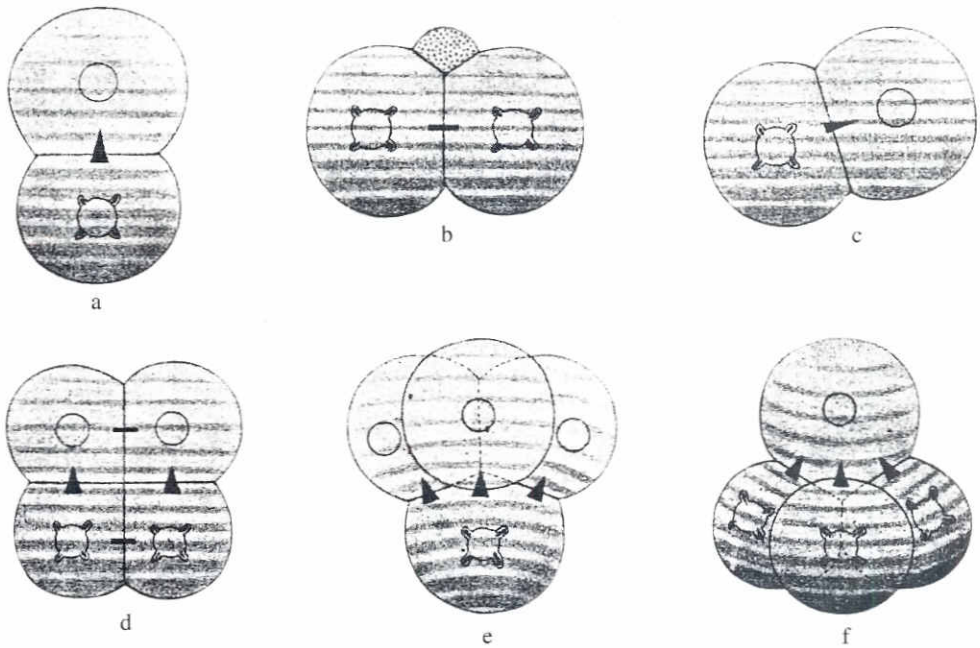


Fig. 11. According to Boveri's 'relativity hypothesis', segregative cleavages occur in four steps: *(i) the mother cell undergoes polarization which necessarily involves the formation of a 'Gefälle' (a graded property), (ii) the spindle rotates by 90° from the equatorial axis into the axis of polarity, hence cleavage division occurs perpendicularily to the 'Gefälle', (iii) the polar organization dissipates in daughter cells, (iv) connected with a change of the cytoplasmic milieu. The quantitative differences in the level of a signal are sufficient to cause cellular bifurcation and alternative behaviors of chromosomes in the arising daughter cells (diminuative behavior in presomatic daughter blastomeres). a normal cleavage of P_0, b symmetrical division parallel to the polarity-axis into two P_1 cells ('ball-embryo'). c If the cleavage plane deviates to some small degree from the polarity-axis, cellular decision-making is always normal. The zygote P_0 goes through segregative division and generates AB- and P_1-daughter blastomeres. This is deduced from the next cleavage which always gives rise to the canonical T stage. Therefore, the polar organization of the zygote cannot persist; neither in AB nor in P_1. The potential to undergo polarization is always distroyed in AB, the germline sister P_1, and its descendants, P_2 and P_3, undergo normal polarization. In addition, in germline blastomeres, the neighboring cells strongly influence the direction of polarization as indicated by spindle orientation in the P_1's of 'ball-embryos' and of types II and III of the dispermic simultanvierer. d type II (2AB's+2P_1's), e type I (3AB's+1P_1), and f type III (1AB+3P_1's) of dispermic simultanvierers. From Boveri (1910b).*

Boveri then discovered that in the untreated zygote an exact same rotation of the MA takes place (see Fig. 4, Boveri 1910a). This crucial observation is rationalized in the seminal concept of 'segregative division' - an alternative to local factors and a priori mosaicism. Boveri extended his notion of autonomous cell-specification by division, by excluding mutual cell-to-cell interaction to maintain a quasi-stable state of two P_1-cell qualities. Killing one of the two P_1-blastomeres of the 'ball-embryo' by UV-irradiation had no influence on the further cleavage program of the other cell leading to a partial embryo. In summary, although no material has been lost, something crucial (AB quality, and hence a major portion of the worm program) is lost from the 'ball-embryo' at the first cell cycle, despite symmetrical division in the centrifuge.

Boveri (1910b, p.195) rationalized the results from both experiments as follows: 'The Ascaris egg, upon its first cleavage division-cycle, can separate into 3/4 AB and 1/4 P_1, or 2/4 AB and 2/4 P_1; or 1/4 AB and 3/4 P_1 (dispermic eggs); or O AB and 4/4 P_1 ('ball-embryo'). This result simply rules out the assumption of any organ-forming regions.' Boveri clearly rejects the dogma of prelocalization, i.e. the presence of specific determinants restricted to a certain cytoplasmic domain in the prototype of mosaic development.

Recently, by applying hydrostatic pressure at the pronuclear stage, centrosome reproduction could be suppressed (Seidl *et al.*, 1988). These non-stratified eggs are unable to undergo cytokinesis (see above). After completion of the first cell cycle, as indicated by their tetraploid state, those eggs, remarkably, adopt either AB- or P_1-quality. In case of AB-fate they divide meridionally (for cleavage pattern of S_1 (AB) and its descendants see Fig. 7), as it is the case during 'ball-embryo' formation at the first (!) cleavage step (Fig. 10). However, at the following cleavage both cells divide synchronously displaying parallel spindle orientation perpendicular to the egg-axis. They both undergo diminution, and their descendants never regain the potential to divide asymmetrically. In other words, the egg after completion of the first cell cycle had changed completely into the somatic AB state and still is a single cell. This result is complementary to Boveri's 'ball-embryo', where P_0 becomes converted to P_1. The complete cell state change of P_0 into either AB or P_1 reinforces the argument against prelocalized cell-fate specifying determinants.

Moreover, Ascaris egg fragments can also regulate into whole larvae, provided that the fragmentation occurred before the first cleavage cycle is initiated. From this finding Boveri (1910b, p. 211) arrived at his own version of the 'harmonious equipotential system': 'An egg and each cell is constructed such that a part of it repeats the dynamic structure of the whole.' In modern terms, Boveri ascribed the quality of 'fractals' to embryonic cells, which accomodate parts as the whole and vice versa. He had a clear vision of cells and embryos as dynamic systems.

Boveri's statement (1910b, p. 211) that 'During cell division an embryo does become a mosaic', in contrast to a single cell, is consistent with the results of classical isolation experiments. It lends no support for the postulate of a promorphological mosaic of determinants in the uncleaved egg or any blastomere derived from it by segregative division. From two related phenomena (i) 'partial cleavage' in sea urchins (progression of the 'developmental clock' in the absence of cleavage divisions), and (ii) partial twinning in Ascaris ('ball-embryo'), Boveri concluded that

embryonic cell-cycles are activation cycles of cell-fate specifying significance. Since normal cellular decision-making may occur even without cytokinesis, he stated (1910b, p.195) 'there must occur in the undivided egg those changes that are usually associated with the step of division, and not division per se would be the decisive step but a process tightly associated with it which, for the time being, we are unable to name.'

At this level of insight Boveri could define essential principles of cellular decision-making in early embryogenesis (Fig. 11). The first event which breaks the symmetry of the egg is a general process of 'polarization'. This was originally assigned to the sea urchin egg, based on his demonstration of an animal-vegetal anisotropy. The polarization event is associated with a polar transport, mostly in animal-vegetal direction, as indicated by the directed movement of pigment granules in the *Paracentrotus* egg, the segregation of cytoplasmic components of different color in *Styela*, positioning of germline associated granules in *Cyclops*, and so on. Polarization leads to an invisible layering of 'something unknown'. According to his 'relativity ('Gefälle') hypothesis' a graded concentration of a property is intimately connected with the polar organization of the egg. Boveri also discussed an 'absolute' hypothesis, an abrupt change in regional cytoplasmic condition, but later he preferred a gradual condition, because cells are never mosaics. This graded property is transient and may diminish, as he deduced from the altered cleavage behavior of aged mature sea urchin eggs which were prevented from insemination for longer times. In the second event (discussed above) the MA rotates in parallel to the polarity-axis, indicating the force-generating activity of the polar cytoplasmic architecture (Figs. 4, 11). This rotation appears to result from a struggle between the two asters for membrane contacts at the animal hemisphere with uncertain outcome as to which aster 'wins'. From the occurrance of duplication ('ball-embryo') and triplication (dispermic eggs) of P_1-blastomeres at bipolar and tetrapolar mitosis, respectively, Boveri reasoned that the centrosomes are not the primary movers of blastomere specification. Rather, sister centrosomes are functionally equivalent. Different activities on opposite poles shift the MA toward the vegetal pole. Therefore, according to Boveri's rule of cell division (cleavage midway between two asters), cleavage produces daughter cells of different size (a large AB and a small P_1). In a third step, concomitantly with the asymmetrical cleavage-division, the polar organization of the cytoplasm dissipates. The different concentrations of the unknown but very general factor leads to the generation of alternate cellular fates. This decision occurs abruptly and very late in the cell cycle, and even in the absence of cytokinesis. Boveri deduced this from two experimental results. First, the fate of the 'ball-embryo' could be reversed still at metaphase of the first cleavage step. Second, occasionally two of the four primary blastomeres after the first cleavage step of dispermic eggs would fuse. This fusion occurs shortly after egg-cleavage. Never did the resulting fusion products show mosaic character, they are either AB or P_1. A strong argument for the 'relativity' in favor of the 'absolute' hypothesis comes from the many centrifugation experiments that did not result in 'ball-embryo' formation. In all cases of centrifuged eggs where the cleavage plane deviated to minimal extent from the polarity-axis (Fig. 11c), the sister blastomeres apparently adopted always alternative normal fates (AB and P_1), and the embryo developed into a normal larva. Obviously, a small initial difference suffices to generate the two alternative normal cytoplasmic states (1910b, p. 198), one with an overall higher or lower level of unidentified 'condition'. This general quantitative and transient condition provides a different environment for the nuclei, directing their activity in alternative directions, one rather immediate marker being chromatin diminution in presomatic blastomeres.

In summary, according to Boveri's concept of autonomous segregative cleavage- division, cell-polarization generates the conditions for cellular bifurcation. This concept raises four fundamental questions: Which properties enable cells to undergo polarization? What factors (cytoskeleton, enzymes, intracellular receptors, etc.) cooperate in generating that polar dissipative structure? What (non-informational) molecule is the main component in shaping that structure? What determines the direction of polarization? Clearly, the early embryonic Ascaris germline, including the zygote P_0, is capable to undergo rounds of polarization. In presomatic blastomeres, with the exception of EMSt, this property is distroyed, probably because of a higher degree of an 'activated state'. Moreover, after skipping its cleavage division, the whole egg may transform into an AB-like cell state. It undergoes diminution, indicative of its activated state. Concomitantly, its capability to undergo polarization is irreversibely distroyed as revealed by its further nonpolar symmetrical cleavages. However, the whole egg may also flip from the P_0-state into the P_1-state which is indicative of the emergence of an unstable state, a 'crisis', typical for excitatory systems.

In normal development AB and EMSt stem from the original animal hemisphere of the egg, and C and D originate from the posterior region of P_2 (see Fig. 7). Hence, reversal of the direction of polarization occurs in P_2. This means, that P-cell quality in the first two cycles is associated with the vegetal cytoplasmic domain, but not in the next two equally asymmetrical divisions. Furthermore, giant eggs which arise from fusion of two eggs are able to develop normally. Obviously, they establish one (new) polarity-axis. In the two P_1-cells of the 'ball-embryo' the direction of polarization is variable and frequently deviates from the animal-vegetal axis. From these observations Boveri (1910b, p. 207) concluded that the polar organization of cells cannot be a preformed quality. Rather: polarization arises as something new. Because the graded polar organization of cells is transient and recurrent, it cannot consist of cell-type specific morphogens (or gradients thereof) that directly control specific nuclear activities at different (i.e. more than one) threshold levels. Boveri's intracellular 'Gefälle' (slope) dissipates at the end of each cleavage step, creating two alternative but each homogeneous states. This concept is not a precursor of the current popular positional information model, except that the 'Gefälle' must be a very general condition necessary to induce cellular bifurcation (not a gradient of positional information). Boveri's concept, which Lewis Wolpert failed to recognize in his review of gradients in embryology (see Sander, 1994), may be relevant in the evaluation of a recent attempt to re-phrase the old prelocalization theory. As applied to the sea urchin by E. Davidson, instead of postulating prelocalized trans-acting factors of smart genes, regulating downstream genes, it is now assumed that the 'local activation' of globally distributed transfactors may guide the cellular specification in the post-blastula stage (see Ransick and Davidson

1993). This, however, only puts the burden on the local activation of control factors and should lead to mixed cellular states. According to Boveri, this does not happen, because segregative divisions generate quasi-stable 'global' cellular states, but never mosaic cells (albeit multicellular mosaic embryos, see above).

Today we have some ideas of how to get 'something from nothing', i.e. still vague concepts of 'self-organization'. It seems that Turing Reaction Diffusion Systems and the far-reaching notions elaborated in S.A. Kauffmans 'The Origins of Order' (1993) may ring in a new era of a 'Physics of Biology' and illuminate the classical embryo as a 'harmonious equipotential system', and solving the current 'two cultures problem'. We may soon experience a modern version of Boveri's 'polarization', perhaps a universal non-triviality in the historical process of embryogenesis. This would lend support to the epigenetic complement of ontogenesis.

Boveri's polarization concept may also give us some clues towards understanding the early cleavage pattern of the sea urchin. The first and second cleavages are meridional (Fig. 6: 13, 16). They are not segregative divisions. But they are by no means merely proliferative. Rather they are also activation cycles, as indicated by the 'partial cleavage' phenomenon. At the third cleavage cycle, the four cells divide equatorially (Fig. 6: 18) This indicates that the blastomeres of the 4-cell stage establish a polar organization strong enough to cause spindel rotation along the animal-vegetal axis. At the 8-cell stage, as a consequence of the dissipation of the polar structure, the four blastomeres of the animal quartet are no longer capable to undergo further polarization. Concomitantly, they can no longer regulate into a whole larva. This is similar to the behavior of the AB-blastomere in Ascaris (Fig. 7). The vegetal blastomeres, however, undergo strong polarization because the most vegetal segment had been excluded from the earlier polarization at the egg stage. This is indicated by the position of the pigment ring in the *Paracentrotus* egg. Consequently, at the fourth cleavage step and only in the vegetal quartet, the MA's are transposed to a very asymmetrical position near the vegetal pole. Hence, the segregative unequal cleavage produces micromeres (see Fig. 6: 21). Their low activated state is expressed by a slow cleavage tempo, as is typical for the germline blastomeres in Ascaris (here evident already at the second cleavage cycle). In conclusion there is no need to postulate specific micromere-determinants (contrary to textbook wisdom). Furthermore, this state may be responsible for the competence of the descendants of micromeres to interact with the higher activated neighbors descended from the macromeres. This process defines short-range induction and may lead to primary mesenchyme ingression and gastrulation.

Early, strong polarization is characteristic of Ascaris, the prototype of mosaic development. Therefore, cell-fate specifications are mainly autonomous and occur early-on and lineally ('zellenweise' in Boveri's term 1905, p.69). In the regulative type of development strong polarization, sufficient to cause segregative cleavage-divisions, occurs later in cleavage. Therefore, blastomeres are specified in layers ('schichtenweise' in Boveri's term). In addition, cell-fate specification takes place conditionally, i.e. by interaction of cells according to their position in the embryo. Another difference between these egg-types concerns the rule of a constant nuclear-cytoplasmic ratio, first derived from the sea urchin embryo. The cell lineages of the mosaic Ascaris embryo follow a 'developmental clock' rather than a size control. The 'clock' indicates a strong cytoplasmic control mechanism of embryonic cell-cycles and dictates rhythm and rate of cell division in the diverse cell lineages. Thus, the clock overrides the influence of the nucleus in determining the nuclear-cytoplasmic ratio.

Despite much progress in cell-cycle analysis in embryos, yeast and tissue-culture cells with molecular and genetic tools, the late period of the embryonic cell-cycle is not the focus of intense studies. No implication for cell-fate determination has been suggested since Boveri. However, recently it has been shown that during the process of polarization there is an unequal distribution of cytoplasmic bound Calcium in Ascaris germline blastomeres (Moritz, 1990). On the other hand, in presomatic blastomeres which undergo equal division, Calcium becomes equally distributed. Moreover, a massive release of Calcium ions occurs at anaphase which apparently is the critical stage of cellular decision-making in Boveri's concept of segregative cleavage steps. Therefore, a Calcium burst may transiently result in different concentrations of free Calcium in the cytoplasm of arising daughter blastomeres. Such Calcium transient could be the 'graded property' in Boveri's 'relativity hypothesis'. This quantitative difference may trigger different ranges of a Calcium-signal-dependant cascade which lead to different cell-fates and to chromatin diminution in the more activated presomatic blastomere. It was further shown that disturbance of Calcium sequestration by Lithium ions results in chromatin diminution in germline blastomeres. Drug experiments indicate that the microfilament-motility system participates in the generation of blastomere asymmetry. Cytochalasin-induced microfilament disassembly distroys the polar architecture in Ascaris germline cells and, concomitantly, causes chromatin diminution at the following cleavage-division throughout the whole embryo. The challenge of chromatin diminution is to define (i) the components of the cascade which mediates the activation of an yet unknown enzyme which, in turn, modifies the HMG1,2 proteins at certain regions in the intercalary chromosome segment, thus preventing their mitotic condensation (these multiple regions are the so-called interstitial chromatin linkers adjacent to the presumptive somatic chromosomes), (ii) the nature of 'diminution factors', (iii) the organization of these factors into diminution complexes, (iv) the resolution of these complexes through the ordered excision of the noncondensed linker chromatin under the stress of anaphase segregation force, and (v) the formation of stable new telomeres of the somatic chromosomes (see Moritz 1993; Müller *et al.*, 1991). In this scenario, the P-granules (in *C. elegans* and Ascaris) and their transient aggregation and dispersion in the germline of the early embryo become indicators for Boveri's concept of polarization, rather than actual determinants of P-cell fate.

So far it seems that the sea urchin embryo displays mainly conditionally cell specifications and Ascaris early cell-autonomous specifications. Nevertheless, Boveri detected cell-to-cell interactions in the early Ascaris embryo as well. First, he observed that the famous T configuration of the 4-cell stage, through a process he termed topogenesis, turns into a rhomboid shape through the movement of the P_2-cell either to the left or to the right. Boveri reasoned that the division of AB is symmetrical

and that the different contributions of the AB daughter cells to either the anterior or the posterior regions of the larva is directed by interaction with the descendants of P_1. Second, he noted a more dramatic impact on topogenesis at the 4-cell stage. Instead of forming a planar rhomb, the cells could also arrange themselves as a more globular tetrahedron where the P_2-cell makes contact with all other cells. In this configuration the antero-posterior axis of the embryo has become rotated by 90° indicating that the EMSt-P_2 direction determines that axis. Boveri succeeded in transforming T-stages into a tetrahedron configuration by centrifugation. Because a left/right asymmetry is set up in the AB lineage already at the 6-cell stage (Fig. 7), the larvae display several left/right asymmetries. Accordingly, the tetrahedron 4-cell stage develops into a *situs inversus*. This clearly indicates that the four descendants of the AB-blastomeres are equivalent and require conditional specifications. The altered timing of chromatin diminution in certain partial embryos (Seidl *et al.*, 1988) and the characteristic invariant cell movements are indications of cell interactions occurring in the early Ascaris embryo. Therefore, the plasma membrane – besides the cell nucleus – is the other important target for signals which can immediately arise after the birth of blastomeres. On the one hand, polar spindle orientation in P-blastomeres may depend on the prior generation of cues which can transiently appear in the process of polarization at certain regions of the cell cortex. The differential movement of the daughter cells, on the other hand, would require differential decoration of their cell surfaces by transmembrane proteins as a consequence of differential activation (see above). Nevertheless, to date very little is known about proteins which guide cell movement and establish intercellular junctions coordinating position and fate of somatic blastomeres.

In summary, Boveri's analysis of Ascaris development established a new paradigm of cell-fate specification in a mosaic egg, without recourse to the prelocalization concept. Indeed, all developmental studies with *Ascaris* and *Caenorhabditis elegans* can be coherently interpreted along Boveri's logic (Moritz and Sauer, 1993).

Concluding remarks

Why is it that Boveri's genius has not been generally recognized, and why have the predictions of his 'relativity theory' not been further tested? One reason may be that his seminal paper was 'buried alive' because it was published in a Festschrift honoring Richard Hertwig and not widely circulated. However, E.B. Wilson (1918, p. 86) was acutely aware of Boveri's reasoning, that his experiments in Ascaris supposedly were inconsistent with the dogma of organ-forming stuff and prelocalized determinants. But Wilson, the life-long friend of Boveri was also a strong adherent of the prelocalization theory because of his own studies and the similar conclusions reached by his student E.G. Conklin in Woods Hole. Wilson failed to include Boveri's theory in his influential book, because he felt that Boveri's stated conclusion, namely the rejection of local factors, was paradoxical, yet only on semantic grounds. His book does contain the crucial experiments performed by Boveri. But from the way in which the dispermy and centrifugation experiments are treated, the reader might indeed conclude that Boveri actually confirmed local determinants as cause for chromatin diminution. This becomes obvious from joint discussion of both kinds of experiments and from the interpretation of cartoons (Fig. 11) that Boveri used – *after* having concluded that there can be no local factors – to visualize his ideas of the *transient* polarization and alternative decision-making. Almost all textbooks (like S.F. Gilbert, Developmental Biology, 4th ed. 1994, and even the otherwise excellent Boveri biography of Fritz Baltzer, 1962) have since propagated this misinterpretation and thus obscured Boveri's conclusive logic (a telling example is the 'documentation of Boveri's determinants' by E. Davidson 1986, p. 443 with Boveri's own cartoons). It seems that the dynamic aspect of the 'relativity theory' has gone unnoticed, in particular the idea of sequential transient states of polarization, bifurcation, and differential activation and repression (see Sander, 1994). Probably the main reason for the demise of Boveri's theory is the deceptively simple modern view that differential gene expression explains autonomous regional and cellular specification because of local or locally activated transcription factors.

Boveri was ahead of his time. In addition to the Chromosome Theory of Inheritance he discovered a developmentally controlled genome-rearrangement (chromatin diminution), and he conducted the first transformation experiment (the merogonic-hybrid experiment), half a century before Frederic Griffith (1928) transformed bacteria. With regard to his epigenetic principles, explaining the emergence of alternate cell states from an almost homogeneous egg-system, he tamed the ugly 'monster of preformation', and, hence, may still remain ahead of our time. Boveri himself might have given the answer why his epigenetic vision – as a complement to his genetic views – has been unappreciated, when he recommends that the old dictum 'doctrina multiplex, veritas una' should be reversed. His reasoning that during development embryonic cells first change their state and then their genome readout, is reminescent of the Copernican revolution, a true paradigm-shift, that could 'polarize' his modern peers into reductionists and holists. If the two cultures problem is ever to be solved, it must realized that life is more complicated than current 'doctrina' has led us to believe.

Boveri also left his mark as a visionary 'organizer' for future fundamental research at the Kaiser-Wilhelm-Institute in Berlin (the fore-runner of to-days Max-Planck-Institute), where he would have been the Director, by selecting four leaders in four developing directions of the biological sciences (Richard Goldschmidt, Max Hartmann, Hans Spemann, and Otto Warburg) two of whom were awarded the Nobel prize.

Boveri was well aware of our limitations in comprehending organisms, because they are products of history. But he was optimistic that progress can be made working like historians. While it is next to impossible to recreate the environment in which the multitude of mostly extinct species evolved (phylogenesis), we can gain some true insight in trying to make sense of those relics common to the many ways in which embryos become adults (ontogenesis). These thoughts are among the many, expressed in poetical language and based on a large canvas of life histories, in Boveri's famous inaugural address as Rector of Würzburg University (1906) entitled 'The organisms as historical beings'. In this work he pays homage to Darwin whom he compares to Copernicus and the first revolution in physical science. Boveri stopped short of comparing Darwin to Newton because in his view Darwin did not yet provide a full mechanis-

tic explanation of the evolutionary process. In retrospect, it seems to the authors of this essay that Boveri has gone beyond Newtonian Mechanics and seems closer to Einstein in proposing his 'relativity theory' of development. We conclude our brief commentary with an evaluation of Boveri's genius by the great geneticist Richard Goldschmidt (1958): 'It seems to me that, as the years pass by and modern biology progresses, Boveri's fame not only will not fade but will shine brighter than ever.' - Let there be light.

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