# Amphibian gastrulation:
# history and evolution of a 125 year-old concept

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The classical characteristics of a gastrula stage in Metazoan development were established by Haeckel (1874, 1875) and since then have been constantly used by embryologists. Different kinds of gastrulae have been recognized in animal groups and invagination phenomena, leading to a two-layered embryo, are considered more primitive than other gastrulation processes (Denis, 1997). At the beginning of the 20$^{th}$ century, mostly under the influence of Spemann's studies and concurrently with early cleavage stages, the Amphibian gastrula became a prevailing model for experimental embryologists (Fig. 1). This led to the discovery of the organizing center by Spemann and Mangold (1924), to which a recent issue of this journal has been devoted. Neural induction during gastrulation was to be investigated for decades. Mesoderm induction at a pregastrula stage was characterized later (Nieuwkoop, 1969).

At the present time, an immense number of articles have dealt with cell inductive interactions in the Amphibian gastrula, a topic that has been renewed during the last decade by the characterization of many genes involved in embryonic prepatterning. But what about the processes leading from a relatively simple blastula to the formation of an advanced gastrula ? Its architecture is now much more complex, with three germ layers subdivided into several domains that are distributed along anteroposterior and dorsoventral polarities. The "morphogenetic movements" widely involved in this construction were described by Vogt (1925, 1929), then studied by Holtfreter (1943, 1944) and modern insights are still emerging (reviews in Keller, 1986 ; Keller *et al.*, 1991), but the determination and the interrelationships of these dynamic processes remain largely unsolved.

This essay will not deal with induction phenomena, which are frequently addressed by detailed reviews. It will deal with the evolution of our knowledge about the mechanisms of Amphibian gastrulation and will include two successive parts. The first and

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longest consists in a historical review of the older studies on Amphibian gastrula, including long-lasting controversial debates about fundamental characters (invagination or delamination, epiboly movements, origin of notochord and mesoderm). A few forgotten pioneers will be celebrated and misconceptions will be mentioned too. In the following part, we shall try to show in which directions our knowledge of gastrulation processes has been evolving during the second half of the 20th century. Table I summarizes important steps in Amphibian gastrulation studies. A prerequisite to the reading of this article concerns the meaning of *invagination* and *involution* to describe the overall process of inward movements in Amphibian gastrulation, leading to archenteron formation and to mesoderm segregation. Already mentioned as a hypothetic movement by Van Bambeke (1870) and Goette (1875) who observed gastrula sections, *involution* (rolling in of a tissue) was used in connection with invagination by Balfour (1881), then described by Vogt (1922a) in the blastopore lips of the living gastrula, but it was still considered as a component of invagination as a whole. This interpretation was maintained during the following decades (Berrill, 1971 ; Balinsky, 1975). More recently, Amphibian gastrulation was described as resulting from invagination *combined* with involution (Grant, 1978). Since involution also affects the deep mesodermal anlagen in Anurans (Keller and Schoenwolf, 1977), it was then considered as a separate process, invagination in the strict sense being applied to the beginning of archenteron formation only. Keller (1981) even wrote :

TABLE I

**MILESTONES IN THE HISTORY OF AMPHIBIAN GASTRULATION**

| Year | Author(s) | Contribution |
|---|---|---|
| 1862 | Stricker | First histological sections through toad embryos at still unnamed gastrula stages. |
| 1872 | Haeckel | Proposes the name « gastrula ». |
| 1875 | Haeckel | Amphibian gastrula included into the series of Metazoan gastrulae. |
| 1875 | Lankester | Introduces the word « blastopore ». |
| 1875 | Goette | Successive gastrula stages and the « marginal zone » are described in *Bombinator* |
| 1882-83 | Hertwig | Comparative description of Urodele and Anuran gastrulation, of notochord, mesoderm and coelome formation. Gastrulation by invagination is accepted. |
| 1888 | Schultze | Hertwig's hypothesis on coelome formation rejected. |
| 1890 | Morgan | Comprehensive analysis of blastopore formation. |
| 1894 | Morgan | Bottle cells are described but not represented. |
| 1895 | Kopsch | First experimental evidence for epibolic and inward morphogenetic movements. |
| 1907 | Ruffini | Describes bottle cells and ascribes them a key role in gastrulation. |
| 1911 | Goodale | First use of vital dye staining on an Amphibian blastula. |
| 1923 a,b | Vogt | First comprehensive drawings of vital dye mark movements. Mesoderm origin from the marginal zone is confirmed. |
| 1925 | Vogt | Details of vital dye mark technique. |
| 1929 | Vogt | Landmark article on gastrulation movements and fate maps. |
| 1943-44 | Holtfreter | Behavior of living gastrula cells *in vivo* and *in vitro*. |
| 1950 | Nieuwkoop & Florschutz | Description of *Xenopus* gastrulation. |
| 1975-76-78 | Keller | *Xenopus* fate maps and morphogenetic movements (radial intercalation). |
| 1983 | Boucaut&Darribère | On the inner side of the blastocoele floor, presence of fibronectin in the fibrillar ECM to which migrating mesoderm cells attach. |

"Most investigators, including the author (Keller, 1975), have erroneously assumed that amphibian gastrulation occurs by invagination.[…].The formation of the archenteron roof from the suprablastoporal endodermal cell sheet is in the nature of a true involution". This opinion might be too radical, though dissecting the complex association of various kinds of cell movements has proved difficult. In the following, I will use the word *invagination* either with its old general meaning as did the former authors, or with its more restricted significance as used in recent articles. I will mention *involution* when it clearly corresponded to a precise description of an "invagination" process, and also, as it is used in more recent articles.

## I. Depicting the Amphibian gastrula: how it forms and how it changes

### The unnamed gastrula

A few authors described Amphibian embryonic development during the first half of the 19th century. They quickly recognized that the lower white hemisphere of the "egg" was progressively covered with the pigmented territory expanding from the upper hemisphere.

Rusconi (1826) thus accurately described the early developmental stages of a green frog egg (*Rana esculenta* ?) and particularly the external appearance of the still unnamed blastoporal slit, which gave rise to an orifice that was considered to be the future anus. At the same time, Dutrochet (1826) proposed a closely similar interpretation, although he still believed the embryo to be preformed in the ovarian egg in the shape of a polyp. Fertilization was supposed to be necessary for constructing a bilaterally symmetric embryo from this polyp form by epigenetic processes. For a long while, later investigators used the term "anus of Rusconi" to designate the blastopore. The correspondence between this orifice and the posterior end of the embryo was briefly mentioned by von Baer (1836), following his description of early development of the brown frog *Rana temporaria* (1834), in which only cleavage and blastula stages were considered. However, von Baer (1837) did not accept the view that the anus itself formed so early. In a reply to von Baer's article of 1834, Rusconi (1836) laid stress on the evolution of the "anal opening" in the newt embryo. He drew sections of an advanced yolk-plug stage, including the newly formed, crescent-shaped intestinal cavity, which he did not interpret as such, although he related these aspects to changes in yolk organization. On the other hand, he mentioned that the intestinal canal was the last organ to be organized. Rusconi also insisted on the fact that, in Amphibians, yolk and embryo are merged into a unitary system, thus differing from the bird egg.

During the following decade, European embryologists extended their observations on Vertebrate development and worked on the germ layer theory as launched by von Baer. Remak (1851), in his treatise of Vertebrate embryology, published enlarged drawings of sagittal sections corresponding to successive stages of frog gastrulae. The material was obtained from embryos of *Rana esculenta* hardened for two days in a mixture of sulphuric acid, alcohol and copper sulphate. The gastrula cavities were correctly depicted. Remak pointed out that the digestive cavity did not originate from the segmentation cavity (now blastocoele) as thought by von Baer, but from the elliptical cavity first described by Rusconi. The true anus formed *ventrally* to the yolk-plug, at variance with Rusconi's view.
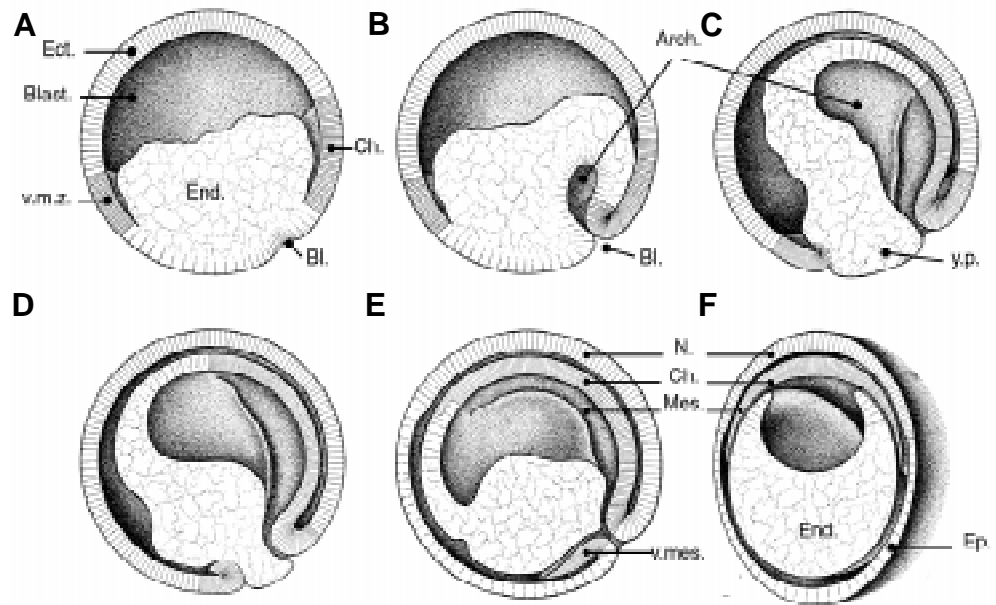
**Fig. 1. Classical interpretation of an Amphibian gastrula** *(adapted from Spemann, 1936).* **(A-E)** *Sagittal sections through successive gastrula stages, from the onset of blastopore formation* **(A)** *to archenteron invagination* **(B,C,D)** *and from yolk-plug formation* **(C)** *to complete resorption* **(E)**. **(F)** *Cross-section through the late gastrula* **(E)**. *Arch., archenteron; Bl., blastopore; Blast., blastocoele; Ch., notochord; Ect., ectoderm; End., endoderm; Ep., epiblast; Mes., mesoderm; N., neuroblast; V.mes., ventral mesoderm; v.m.z., ventral marginal zone; y.p., yolk-plug. The discontinuity between dorsal endodermal ridges and dorsal mesoderm* **(D to F)** *has been challenged by later studies.*

But Remak made two important misinterpretations concerning the early germ layers. First, the dorsal wall of an early gastrula included all three germ layers. The external layer associated two cell sheets, respectively an external brown one and an internal clearer one. The middle germ layer, spreading all around the animal hemisphere, was formed of very small cells and its meridian dorsal part gave rise to the notochord. It was described as segregating from the external germ layers and becoming inserted between them and the internal "trophic layer" (now endoderm), made up of large cells. Moreover, Remak believed that the digestive cavity (later named archenteron) was entirely formed inside the internal layer, originating from the white hemisphere that invaginated into itself, like the intestinal tube of the chick embryo : "the lower surface of the frog egg turns into the inner surface of the alimentary tube" (Remak, 1852).

The superposition of the three germ layers in the animal hemisphere as described by Remak remained a valid interpretation for a long time, as we shall see later. The distinctive characters established by Remak between the three germ layers (sensorial, motor and trophic or glandular) were sucessfully used during the following decades, but the interpretation of the gastrula stage was still far from being possible.

Ten years later, Stricker (1862, 1864a) devised for the first time a histological technique for making sections through *Bufo* eggs or embryos that had been embedded in a mixture of stearin and white wax. This technique allowed him to draw detailed unstained sections through blastulae and gastrulae, but instead of analyzing the previously assumed invagination process, Stricker interpreted the observed structures as originating from separations between cell groups, leading to inward delaminations. Delamination was also considered responsible for the appearance of several dorsal cell layers. Stricker rightly interpreted Rusconi's elliptical cavity as being intestinal.

In 1867, Van Bambeke submitted to the Belgian Royal Academy a much more detailed article on the development of the spadefoot toad, *Pelobates fuscus.* He analyzed as completely as possible the complex cell layer structures that he found in the roof of the animal hemisphere. He carefully compared his observations with those of

Stricker and Remak, and tried to characterize the germ layers according to the latter's definitions, which did not always fit to his own findings (Fig. 2A). Van Bambeke believed that no true invagination occurred into the vegetal hemisphere, but that limited involutions giving rise to the dorsal (Rusconi's) and ventral (Remak's) anal cavities, were prolonged inwards by delamination processes. Van Bambeke's work (1870) long remained a reference for anuran development, since it encompassed most of the stages between the ovarian egg and the hatched tadpole, and it was illustrated with beautiful histological sections.

At approximately the same time, in Germany, Goette (1869) published his first observations on the development of the fire-bellied toad, *Bombinator igneus* (now *Bombina bombina*), which he wanted to choose as an anuran model species for comparative studies. We shall consider the early developmental stages only (including the gastrulation processes), for which he compared his own observations with Remak's interpretation. He admitted that the primitive intestinal cavity formed consecutively to an invagination process, but opposed Remak's view on the formation of germ layers. Remak believed that the middle layer arose from the blastocoele roof, whereas Goette maintained that it was derived from the yolky inner layer. His drawings are schematic and show three germ layers in the dorsal roof of middle and late gastrulae, of which the inner layer is always distinctively one-cell thick. These illustrations were later redrawn for an atlas, on which we shall comment. Goette had also investigated fish, bird and mammalian embryos and first wanted to compose a treatise of comparative Vertebrate embryology, but he turned to writing a monograph of *Bombinator* development from the ovarian egg to the metamorphosed toadlet. There he compared the characteristics of *Bombinator* with those of other known Amphibians and Vertebrates. This huge book (more than 950 pages !) was illustrated with a beautiful atlas of 22 plates, including 380 figures. It long remained an embryological and anatomical reference book,. being cited for decades (Goette, 1875).

Before we come back to Goette's embryological studies, it is necessary to evoke the birth of the gastrula concept in Ernst Haeckel's works at the same time.

### The gastrula concept (Haeckel, 1872)

Haeckel is widely known as a prominent early supporter of Darwin's evolutionist theory. In the late 1860s, he was trying to draw a phyletic tree of the animal and vegetal kingdoms and was especially interested in embryological comparisons between different phyla, inasmuch as they shared similar embryonic stages. This was considered to be evidence for phylogenetic relationships between them. The early similarities between otherwise different animals were thus believed to prove their relationships. In 1866, in the second volume of his book *Generelle Morphologie der Organismen*, devoted to general developmental and evolutionary features, Haeckel had written his famous sentence : "Ontogeny is the short and rapid recapitulation of phylogeny", which was later incorporated into his "fundamental biogenetic law". The developing individual organism was supposed to go through the morphological and physiological steps that had characterized its ancestors. Haeckel, a few years later, came to the conclusion that, among these steps, a structurally simple and early embryonic shape was common to all Metazoans. He named it *gastrula* (Haeckel, 1872). It was described as a hollow diploblastic ovoid stage of development, whose internal digestive cavity was limited by an endodermal cell layer and opened at one pole : the orifice was interpreted as a primitive mouth (*Urmund* in German, a word that was still long used by German authors, though Ray Lankester (1875) proposed to name it *blastopore*, describing the *invaginate planula* as an equivalent of Haeckel's gastrula). Such a simple structure was found by Haeckel among marine calcareous sponges, whose embryonic life displayed a gastrula step, preceded by *morula* and *blastula* stages. Haeckel compared their development with that of various marine Invertebrates that had been studied earlier by several authors (*Sagitta;* sea-urchins and other Echinoderms ; Ascidians ; *Amphioxus*, a chordate that was still considered a primitive and "skulless" Vertebrate, etc.). A comparative classification of different types of *gastrulae* was then established by Haeckel (1875). Lankester himself(1875) published a similar classification, using the word *planula* instead of *gastrula* and, on the following year, he wrote an account of Haeckel's article for English readers (Lankester, 1876). He then proposed to name *archenteron* the primitive intestine. Lankester also currently used the words *ectoderm* or *epiblast*, *mesoblast*, *endoderm* or *hypoblast*, to name the cell layers and these names were widely used subsequently. He had also proposed the distinction between diploblastic and triploblastic Metazoans (Lankester, 1873).

Haeckel's article on the different kinds of gastrulation had been preceded by a landmark article in which he proposed a new evolutionist concept derived from the real gastrula, namely that of *gastraea* : it was supposed to have been a primitive organism from which all Metazoans had evolved, retaining the gastrula stage in their individual history as a phylogenetic memory of their ancestor (Haeckel, 1874). It would be outside the scope of this review article to evoke the multiple controversies that arose for years between Haeckel and his numerous critics. They were recently discussed in detail by Rupp-Eisenreich (1996a), who especially devoted a long analysis to the embryological polemics.

Haeckel (1875) used investigations from earlier authors to document the gastrula characteristics of Amphibians and other Vertebrates. Since the primitive means of gastrula formation was supposed to be an invagination process, giving rise to a diploblastic structure from a hollow cellular ball (blastula), he paid homage to the genius of Remak, who had described the Amphibian gastrula as forming by invagination. However, being dissymmetric, the formation of the digestive cavity introduced a dorsal-ventral polarity in the embryo and the gastrula was no longer monaxonic in Amphibians: the earlier bilateral symmetry of the frog egg had not yet been described at that time. Haeckel cited Goette's work and referred to his figures (Fig. 2 D,E). We shall return now to the analysis of Goette's contribution to the description of *Bombinator* gastrulation.

In this book, Goette (1875) compared the cell sizes in blastulae and early gastrulae and came to the conclusion that, at the level of the blastocoele floor, animal external cells were contiguous to a marginal zone (*Randzone*), composed of slightly larger cells that were internally connected with the mass of large yolk cells. This was the first mention of the gastrula marginal zone, a term that is still widely used. Although this transitional region had no accurate limits, the author emphasized that it was characteristic : he described it as swelling at the onset of blastoporal slit formation, pushing the large yolk cells upwards. Goette believed that the involution movement of the blastopore lip was only apparent, actually corresponding to growth of cell groups pushing inwards and forwards. On the dorsal side, the separation between the external primitive layer (ectoderm) and the secondary layer (endoderm + mesoderm) was provided by the marginal zone and gave rise to the blastopore slit (Fig. 2 D,E). The secondary layer segregated into middle and internal layers (respectively mesodermal and endodermal), but, due to their nutritional role, the largest yolk cells were not considered as belonging to the endodermal layer, which only delimited the digestive cavity. Goette admitted that archenteron formation could be interpreted as resulting from an invagination process : the yolk cells are engulfed close to the segmentation cavity that progressively shrinks as the archenteron develops and the marginal zone forms a continuous concentric belt. Goette nevertheless believed that there were no true involution movements. If yolk cells were less bulky, the Amphibian embryonic structures should correspond to those of Invertebrates, especially to *Amphioxus*, and there should be an Amphibian gastrula stage. Thus Goette, who discussed at length Haeckel's gastrula concept, did not adopt that same word for *Bombinator* embryos. Of course, in the same year, this was done by Haeckel himself, who reproduced two of Goette's figures in his own article (Haeckel, 1875). For Haeckel, there was no question about the reality of invagination processes in the Amphibian embryo. It must be added that Goette was reluctant to admit Haeckel's theories without criticism and he preferred detailed embryological studies and physiological interpretations to phylogenetic generalization (Rupp-Eisenreich, 1996b).

To close this chapter, we shall consider another work carried out on *Bufo* and *Pelobates* eggs by Moquin-Tandon (1876), who clearly was not yet aware of Haeckel's theories. He mentioned that he had just heard about Goette's book on *Bombinator* but had not been able to find it before publishing his own results. Neither was he aware of the gastrula concept. Moquin-Tandon nevertheless carefully discussed the interpretations of older authors, including Goette's (1869) article. He drew sections of gastrulating embryos that were less schematic than those of Goette and the preceding authors (Fig. 2 B,C). His interpretation of structural embryonic changes does not agree with Remak's invagination

hypothesis. Like Stricker (1862), he thought that the blastoporal slit and archenteron cavity formed by delamination.

Moquin-Tandon also disagreed with Van Bambeke (1870) about the segregation of the different cell layers. This was a very complex topic and such discrepancies between different authors are quite understandable, all the more so because they worked on histological sections and, occasionally, compared them with living, rather dark embryos. The dismissal of invagination by Moquin-Tandon is more surprising, since he admitted, following observations by Stricker (1864b), that yolk cells from the blastocoele floor were able to migrate upwards and to adhere to the inner dorsal side of the blastocole roof, as seen in histological sections (Fig. 2 B,D). This issue had been questioned by Romiti (1873), one of Stricker's Italian collaborators in Vienna. His observations on *Bufo* gastrula cell layers were at variance with those by Van Bambeke (1870). On the other hand, it was not possible to decide between an active displacement of yolk cells or a passive pushing movement induced by cell divisions and volume increase, or by invagination.

Such an opposition between defenders of invagination and of delamination in gastrulation processes was to last for a long time, in spite of Remak's and Haeckel's earlier choice.

### Gastrulation and formation of germ layers

The above-mentioned studies had all been done on Anuran embryos. Several problems had now to be tackled to understand gastrula formation and completion. The origin of germ layers was controversial, especially that of the mesoblast. Was the origin of the archenteron roof, which gave rise to notochord, endoblastic or ectoblastic? How did the thick cellular folds that surrounded the blastopore evolve?

The first comparative studies aiming to solve these problems were carried out by two American authors, Scott and Osborn (1879), in the common newt (*Triturus taeniatus*) embryo. They undertook a comparison between the development of this Urodele and that of *Bombinator,* as studied by Goette. Working in Balfour's laboratory at Cambridge University (U.K.), they accepted the invagination process in Amphibian gastrulation, as explicited two years later by Balfour himself in his treatise of comparative embryology : "The growth inwards of the dorsal wall of the mesenteron [= archenteron] is no doubt in part a true invagination […]. The mesenteron is at first a simple slit between the yolk and the hypoblast, but as the involution of the hypoblast and mesoblast extends further inwards, this slit enlarges, especially at its inner end, into a considerable cavity" (Balfour, 1881, p. 102). At variance with Goette in *Bombinator*, Scott and Osborn found in *Triturus* that the mesoblast was not continuous across the middle dorsal line, but was separated into two lateral plates by the notochord anlage. The latter was itself continuous with the dorsal walls of the archenteron, forming a groove before separating from them (cf. Fig. 4B). Thus, the notochord originated from the invaginate hypoblast, the yolk cells giving rise to the remaining digestive hypoblast (= endoderm). In *Bombinator*, Goette had described a continuous mesoblastic layer between ectoblast and hypoblast, segregating into notochord and lateral plates along the middle dorsal area. Another difference between *Triturus* and *Bombinator* was that, in the latter case, ectoderm was formed of two cell layers, whereas it was still monolayered in *Triturus* at the same stage. The observations on notochord formation were confirmed and extended by Van Bambeke (1880) in other newt species and in the axolotl.
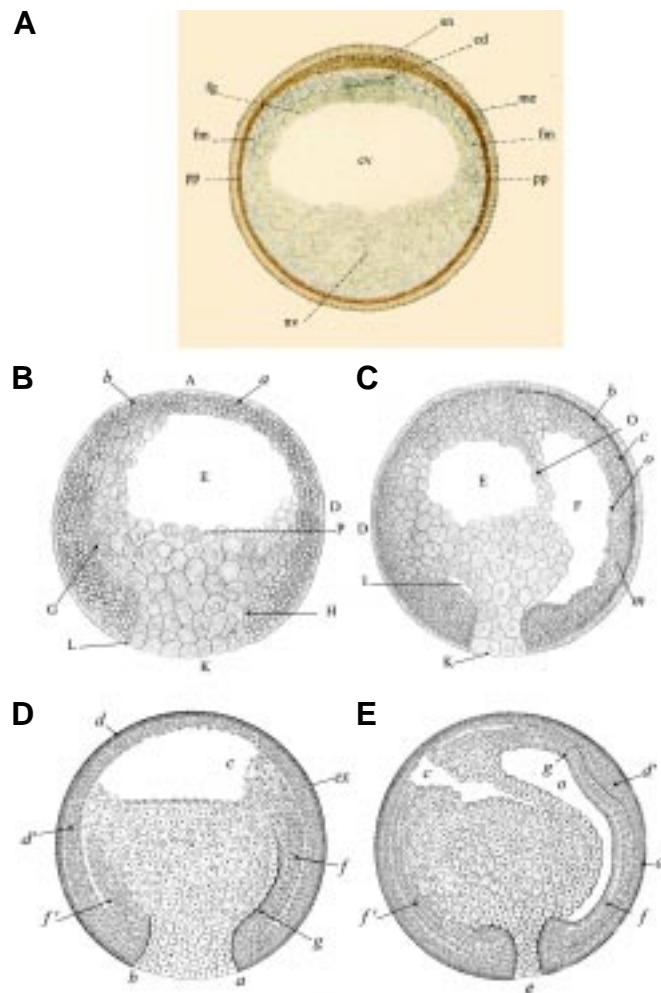


**Fig. 2. Sections through still unnamed gastrula stages of various Amphibians**. **(A)** *Pelobates. Cross-section through an advanced stage (Van Bambeke, 1870). Cd, notochord; cv, 'primitive visceral cavity' (archenteron); fg, 'glandular layer' (endoderm); fm, 'motor layer' (mesoderm); me, externalmost layer (ectodermal); nv, vitelline mass; pp, peripheral part of the 'sensorial layer' (ectoderm); sn, thickened part of the 'sensorial layer' (neural anlage).* **(B,C)** *Sagittal sections through two successive gastrulation stages in Bufo (adapted from Moquin-Tandon, 1876). A, dark (animal) pole; a, 'main layer' (ectoderm external layer); b, 'horny layer' (ectoderm); c, neural layer; D, ventral (sic) side; E, segmentation cavity (blastocoele); F, visceral cavity (archenteron); G, dorsal slit preceding visceral cavity formation; H, ventral slit preceding anal cavity formation; I, anal cavity; K, yolk-plug; L, blastopore; m, 'motor layer' (mesoderm); O, separation between segmentation and visceral cavities; o, 'trophic layer' (endoderm); p, floor of the segmentation cavity.* **(D,E)** *Sagittal sections through two successive gastrulation stages in Bombinator (Goette, 1875; by permission of the British Library, London). Dorsal side on the right; a,b, dorsal and ventral blastopore slits; c, segmentation cavity; d, external layer of ectoderm; d', internal layer of ectoderm* **(D)** *or cerebral plate* **(E)***; e, yolk-plug; ex, ectoderm; f,f', intermediate layer (mesoderm); g, intestinal layer (endoderm); o, intestinal cavity.*

Much more detailed studies were then issued by German authors, in an attempt to produce a general theory of germ layer and coelome formation. Oskar and Richard Hertwig had been former collaborators and followers of Ernst Haeckel in Jena.
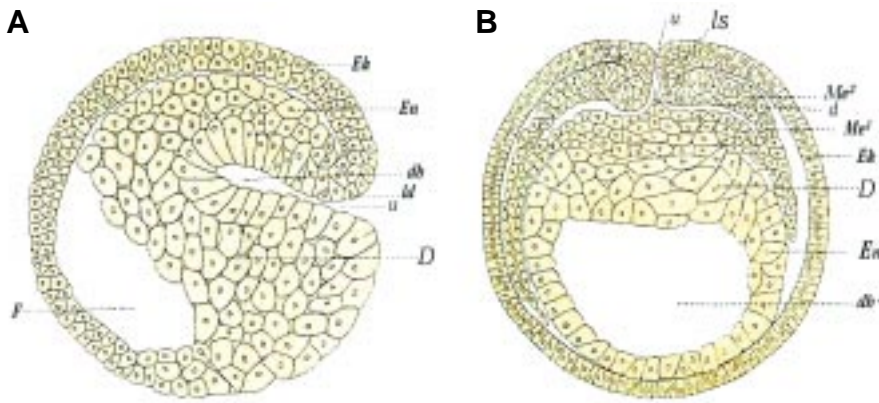
**Fig. 3. Sections through *Triturus* gastrulae** *(adapted from Hertwig, 1882).* **(A)** *Sagittal section, beginning of archenteron formation. This figure became classical and was reproduced many times in several handbooks. D, large yolk-cells; dh, archenteron; Ek, ectoderm; En, endoderm; F, segmentation cavity; ld, dorsal blastopore lip; u, blastopore.* **(B)** *Frontal section through an advanced gastrula. D, Ek, En, u: see (A); d, yolk-plug remnant; dh2, enlarged anterior part of the archenteron; ls, lateral lip of blastopore; Me1, visceral layer of mesoderm; Me2, parietal layer of mesoderm.*

Together they published a long article on the coelome theory and the problem of mesoderm formation in Metazoans, the 5th chapter of which (pp. 53-67) was devoted to Vertebrates (Hertwig and Hertwig, 1882). The main points dealt with differentiation of tissues and organ systems from mesoderm anlagen. Simultaneously, Oskar Hertwig alone published a still longer analysis of mesoderm development in Vertebrates, mostly in Amphibians (Hertwig, 1882, 1883). The first article was entirely devoted to the Urodele *Triturus taeniatus*, preceding a comparative analysis of the situation in the frog *Rana temporaria* in the second article.

Hertwig's drawing of a sagittal section through a newt gastrula was reproduced later in numerous books (Fig. 3A). It is fairly schematic (Hertwig, 1888, Fig. 41, and 1906, Fig. 398), but shows the presence of elongated archenteric cells, actually the first representation of bottle cells, on which Hertwig did not make significant comments. In the first article (1882), Hertwig also described the successive steps of newt gastrulation with more details than his predecessors. He understood that animal cells were continuously moving towards the vegetal pole during invagination, with an increase of cell membrane surface inside and outside. Hertwig definitively discarded the former theories about mesoderm formation by delamination of one of the primitive germ layers, ectoderm or endoderm. Mesoderm originated from two cell groups appearing in the blastopore region on both sides of the "chordendoderm" (dorsal roof of the archenteron giving rise to the chordal anlage). They progressively stretched out over the inner surface of ectoderm and the outer surface of endodermal yolk cells, growing ventralwards from the dorsal region (Fig. 3B). The immediate surroundings of the blastopore and the chordendoderm margins were the only area in which mesoderm anlagen could not be distinguished from adjacent cell layers. A proliferating cell zone, probably originating from yolk cells, could be found in this region. Since the two cell sheets (parietal and visceral) of each mesodermal layer were quickly separated by the coelomic cavity, Hertwig proposed that the mesoderm anlagen might originate from endoderm evagination, as in some Invertebrates (sea-urchin). On the other hand, Hertwig confirmed the separate formation of notochord from the chordendoderm, originating from involuted ectoderm cells (Fig. 4), as seen by Scott and Osborn (1879).

In the second article, Hertwig (1883) studied the gastrulation process in the brown frog (*Rana temporaria*). He mostly found similarities between newt and frog mesoderm formation, with

secondary differences that he attributed to cell size and pigmentation, which could blur cell layer distinction. He therefore rejected several of Goette's interpretations on mesoderm formation and evolution. The archenteron roof included the chordendoderm, which originated from dorsal involuting ectoderm cells. Somitic and lateral mesoderm formed from two posterior anlagen growing dorsalwards and developing from paired posterior invaginations of the archenteric cavity. There were thus three invagination cavities, the first one corresponding to the archenteron, the other two being mesodermal and opening in the internal part of the blastopore. The schematic drawings of such an interpretation were long reproduced thereafter (Hertwig, 1888, Fig. 62 and 64 ; Hertwig, 1906, Fig. 309) since Hertwig assumed that, even if the coelomic cavities were not at first visible between the two mesodermal layers, they were virtually present at the beginning of mesoderm formation. This hypothesis was a Haeckelian attempt to obtain a nice comparison of mesoderm formation between frog and *Amphioxus*, the latter being still considered a primitive Vertebrate. Nevertheless, mesoderm cell pigmentation favoured an ectodermal rather than an endodermal origin.

Hertwig refused to admit a homology between the grooved early medullary plate in the newt and a primitive streak, as proposed by Van Bambeke (1880), imitated by several others during the following decades.

The first edition of Oskar Hertwig's treatise of embryology of man and Vertebrates was issued in 1888, and nine others followed until 1915. Hertwig reused and systemized the results he had published in the above-mentioned articles (1882, 1883) and this celebrated text-book was translated into English and French. The controversial results obtained by other authors on the same topics had no influence on the rigidity of Hertwig's initial views. Some of the first discrepancies about gastrulation events were soon pointed out by Schultze (1888) in the brown frog *Rana temporaria*. According to him, there was no characteristic diploblastic gastrula stage in *Rana* : the mesodermal and endodermal anlagen formed simultaneously during archenteric invagination. Mesoderm and dorsal archenteron roof developed from the ectoderm and the three germ layers were intermingled in the blastopore's dorsal lip. In the lateral and ventral parts of the blastopore, the external ectodermal cell layer (*Deckschicht*) was continuous with the endoderm and the internal ectodermal layer (*Grundschicht*) became gradually mesodermal. There was no pair of mesodermal anlagen, because there

was no chordendoderm and the notochord segregated from the mesodermal mantle (Fig. 5A). Hertwig's "coelome theory" therefore did not apply to the frog embryo. On the other hand Schultze accepted that a primitive streak with a primitive groove formed in the posterior half of the late gastrula by fusion of the ectodermal and mesodermal layers (Fig. 5B). Two lateral halves of the neural plate were separated by the primitive groove. Here, a preconceived idea to find an early close homology between frog and bird development is quite conspicuous. Schultze concluded his article cautiously, stating that a unitary interpretation of germ layer formation in Vertebrates was still far beyond reach. His relevant observations would have to be repeated by other authors.

In the axolotl, Houssay (1890) believed that gastrulation proceeded by splitting and delamination, the initial invagination being limited at the time of blastopore formation. There was no epiboly, in the sense that the smaller animal cells did not cover over the vegetal cells. Houssay otherwise confirmed Schultze's views on the origin of the mesoderm, and rejected Hertwig's theory. He admitted the existence of a primitive streak in Urodeles. As in Anurans, notochord originated from dorsal mesoderm, according to Goette (1875).

Perenyi (1891) rejected Hertwig's views about mesoderm formation. He studied *Bombinator* gastrulae and came to an interpretation that he believed more simple than those of his predecessors. He rightly believed that gastrulation involved both epiboly and emboly phenomena. A duplication of the original number (three) of cell layers present in the blastocoele roof occurred above the blastopore and gave rise to notochord (from the most external cell layer), to endoderm (laterally) and to mesoderm (laterally, between endoderm and the superficial ectodermal layer). Such a simplification overlooked detailed structures. Perenyi too proclaimed the presence of a primitive streak at the floor of the posterior medullar groove.

In the same year, Robinson and Assheton (1891) published a long and relevant article on the brown frog *Rana temporaria*, in which they studied the formation and fate of archenteron, the origin of germ layers and notochord, and the establishment of a primitive streak. Although they drew detailed sections, representing invididual cells, they did not pay attention to the presence of endodermal bottle cells. They agreed with Houssay (1890) on archenteron formation by splitting. They stated that there was no invaginate epiblast inside the archenteron. Mesoderm formed from endoderm by delamination but, at variance with Schultze, they described the notochord anlage as indistinguishable from both hypoblast and mesoblast. They nevertheless correctly stated, unlike Hertwig, that the coelomic cavity was formed by internal splitting of the lateral mesoderm.

Criticized by Morgan and Tsuda (1894), Assheton (1894) discarded his own earlier interpretation and admitted that a limited invagination occurred at the beginning of gastrulation.

Other different and original views were exposed by Lwoff (1894) in a very long article dealing with *Amphioxus*, the lamprey *Petromyzon*, the axolotl, the brown frog (*Rana temporaria*), two fishes and the lizard. According to Lwoff, gastrulation meant gut formation only and excluded any other invagination process. In Urodele Amphibians, dorsal "invagination" gave rise to an anlage common to notochord and mesoderm, the so-called "dorsal plate", which originated from ectoderm. It formed the dorsal roof of the archenteron. Again, in Lwoff's view, the ventral endodermal
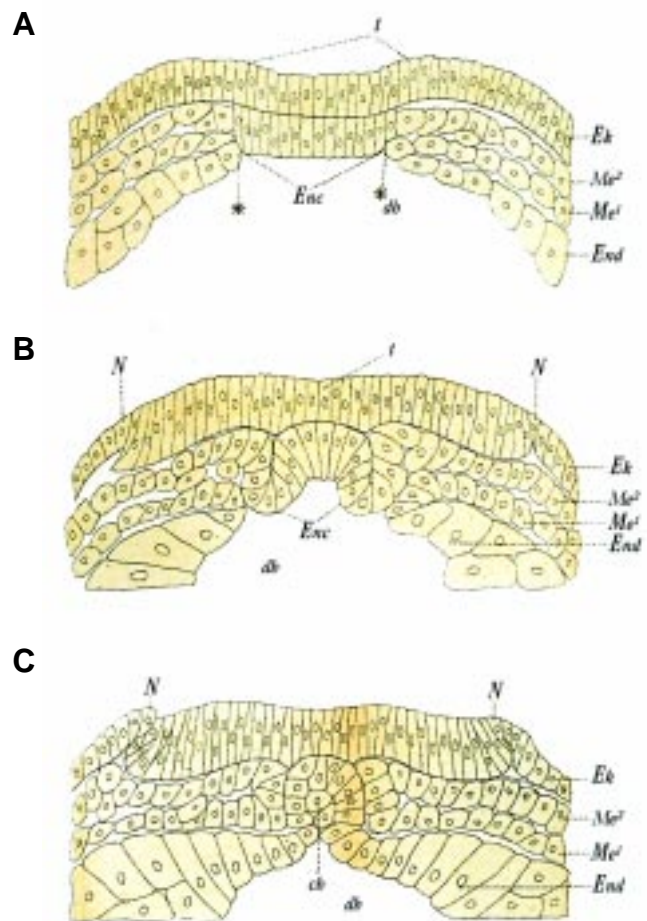
**Fig. 4. Dorsal cross-sections through early *Triturus* neurulae** *(adapted from Hertwig, 1882).* **(A)** *Onset of neurulation; chordal plate between endoderm ridges and dorsal mesoderm anlagen.* **(B)** *Formation of a chordal groove.* **(C)** *Endoderm ridges unite under the notochord anlage separated from somitic mesoderm. ch, notochord; dh, archenteron; Ek, ectoderm; En, endoderm; Enc, 'chordendoderm' (chordal anlage); Me1 and Me2: see Fig. 3B; N, neural folds; t, medullary groove. The asterisks in (A) indicate the junction between endoderm and the visceral mesoderm layer on both sides.*

part of the archenteron was already in place and did not invaginate ; there was no direct correlation between the forward movement of anterior endodermal cells (near the blastocoele) and the involution of ectoderm cells. The archenteron cavity originated from anterior endoderm movement and not from dorsal "invagination", two processes that had been previously merged. The initial cavity was not the definitive intestinal cavity that would be reorganized later. The mesoderm anlagen were formed by separation from the notochord and not by segregation from the posterior endoderm, as thought by Hertwig. In Anurans, a layer of dorsal archenteric cells was already present under the notochord, as seen by Goette (1875) and Schultze (1888). The early dorsal continuity of the endoderm layer in Anurans, as opposed to its interruption in Urodeles, was considered to be an early developmental acceleration with phylogenetic significance. Lwoff also
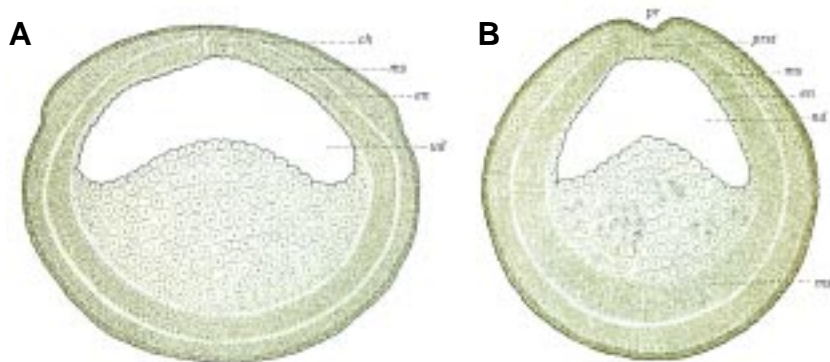
**Fig. 5. Cross-sections at a posterior level through late *Rana* gastrulae** *(adapted from Schultze, 1888).* **(A)** *Gastrulation is completed. The external ectodermal cell layer is very conspicuous.* **(B)** *At a slightly earlier stage (very small yolk-plug), the notochord anlage is not yet segregated and a 'primitive streak' is described. ch, notochord; en, endoderm; ms, mesoderm; pr, primitive groove; prst, primitive streak; ud, archenteron.*

discussed at length the "blastopore theory" and gastrulation events in other Vertebrates.

Studying gastrulation in yolk-rich eggs of Gymnophione Amphibians, Brauer (1897) brought new evidence for dorsal involution processes (Fig. 6). A thick mesodermal layer, giving rise to notochord in the median plane, was described as originating from a superficial " invagination" affecting the primary ectoderm. It could be completed by splitting processes in the anterior endodermal cells. Mesoderm was not of endodermal origin.

Still other authors worked on Amphibian gastrulation between 1875 and 1895 and it is impossible to cite them all.

Working on the influence of the yolk mass on gastrulation in Vertebrates, Samassa (1895) classified the various interpretations of gastrulation events. The whole archenteron was formed from vegetal cells according to Moquin-Tandon, Houssay, Robinson and Assheton, and others. But the dorsal archenteron roof originated from an involution of animal cells, according to Scott and Osborn, Van Bambeke, Hertwig, Schultze, Perenyi, Lwoff, with divergent views about whether or not mesoderm formed from these animal cells. The origin of ectodermal cells, exclusively from animal cells or with a late contribution of vegetal cells, was also controversial. And Samassa erroneously thought, with Roux (1888, 1905), that the neural tube was situated on the originally vegetal part of the egg (Fig. 7) (Sander, 1991).

We shall now refer to Thomas Hunt Morgan's opinion, in his book on frog development that appeared two years later : "I have without hesitation set aside those accounts where the author has transparently sought to find his preconceived theories demonstrated in his drawings of the sections of the embryo"(Morgan, 1897, p. 69). Morgan himself agreed with Schultze (1888) on mesoderm formation processes in the frog and disagreed with

Hertwig's interpretation, but he was ready to accept that notochord could originate from a dorsal evagination of endoderm in Urodeles. At an earlier stage, mesoderm was described as forming a marginal ring under the surface prior to blastopore formation. This was a prophetic view concerning the anuran embryo, close to Goette's former findings (1875). Morgan clearly described the beginning of invagination process for blastopore formation, with cells that "pull away from the surface". Morgan also paid attention to earlier results from Pflüger (1883). This German author, working on the effects of gravity in Amphibian embryo development, had described complex changes in embryo orientation during gastrulation. He noted that the primary vertical axis of the unfertilized egg is modified from the beginning of gastrulation on, when invagination progressively displaces the yolk mass and the blastopore moves towards the initial vegetal pole. These observations unfortunately led to the above-mentioned erroneous assessment about neural tube formation in the originally vegetal hemisphere, adopted by Roux (1888, 1905) and by Morgan (1894). Hertwig (1892) too "grudgingly concurred with Roux's interpretation" (Sander, 1991) . "Schultze's conclusion that the embryo lies over the black hemisphere may be dismissed as it is completely contradicted by well determined facts" (Morgan and Tsuda, 1894). Roux and Hertwig had obtained frog embryos in which a large blastopore remained open between the two cephalic and caudal dorsal parts, each including nervous system and notochord. The occurrence of such anomalies (*asyntaxia medullaris*, *spina bifida*) had led the German authors to reject the previously accepted view that the embryonic axis formed in the animal hemisphere. Localized injuries obtained by pricking the blastopore region also induced developmental abnormalities that Roux and Morgan interpreted as evidence that the embryo forms in the vegetal region of the egg and "that the head

**Fig. 6. Sagittal section through a *Hypogeophis* gastrula** *(from Brauer, 1897). az, cylindric cells originating from the animal external layer; r, limit between involuting and internal cells; ud, archenteron; ur, blastopore lip; vz, internal yolk-cells.*
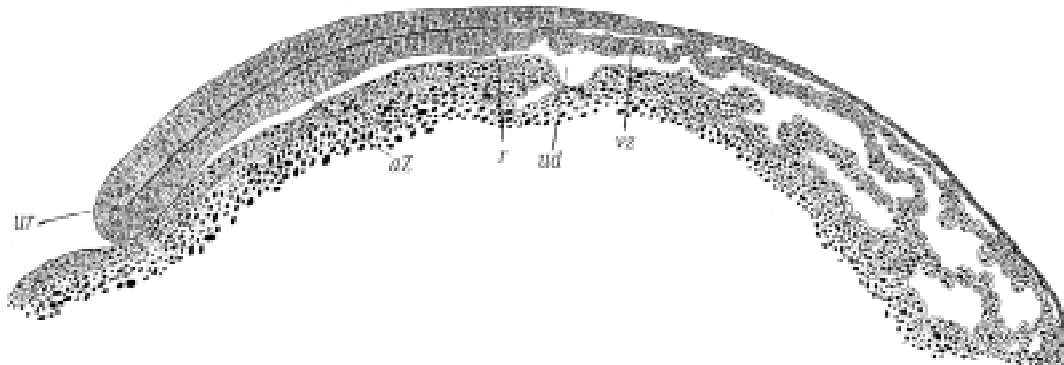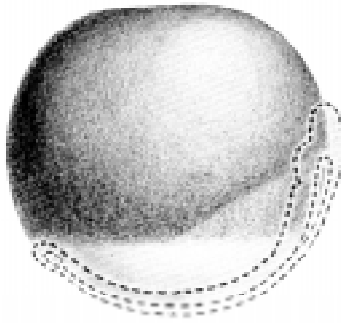
**Fig. 7. Formation of the frog embryo on the egg vegetal hemisphere, according to Roux (1905).** *The head extremity coincides with the grey crescent area.*

end of the embryo corresponds to a point just in front of the position occupied by the dorsal lip of the blastopore when it first appeared" (Morgan, 1894). This erroneous interpretation (Fig. 7) was first corrected by Kopsch (1895a, b, 1900), as we shall see later. Morgan (1893) had nevertheless proposed alternative interpretations to Roux's and Hertwig's experimental teratological results and dismissed the anteroposterior concrescence theory.

### Blastopore formation and evolution

Though the older authors had considered that the blastopore directly gives rise to the anal opening, this opinion was quickly dismissed in the 1880s. It was then believed that, at least in Anurans, the definitive anus originated more ventrally from a secondary intestinal evagination or/and an epidermal invagination after blastopore closure (Goette, 1875). The situation was different in Urodeles, where Johnson (1884) in the newt, then Houssay (1890) in the axolotl, claimed that the blastopore directly transformed into the definitive anus. A general review of the subject was given by Morgan (1890), long before he became a founder of modern genetics. Aged 23, Morgan was then an advanced student in W.K. Brook's laboratory at Johns Hopkins University in Baltimore. Before defending his thesis on Pycnogonids in 1890, he had investigated blastopore changes in two Anurans (*Bufo* and *Rana*) and one Urodele (*Ambystoma*). In the latter case, he concluded that "the anterior portion of the blastopore becomes the neurenteric canal, and the posterior end the permanent anus of the adult". A similar conclusion had already been reached in Hertwig's laboratory by Schanz (1887) in *Triturus*, but Morgan did not mention this work. In the Anurans, Morgan confirmed that the dorsal part of the blastopore becomes the neurenteric canal, the ventral part is obliterated and the anus opens more ventrally. Morgan illustrated his findings with carefully detailed drawings and analyzed more than 25 earlier articles on the situation in Vertebrates. He also discussed phylogenetic problems concerning the functional significance of the communciation between intestinal and neural cavities via the neurenteric canal.

In that same year in Germany, Erlanger (1890) published observations on Anuran blastopore fate, in *Rana*, *Bufo* and *Bombinator*. He too established that the anus was a secondary opening, originating from both an archenteric diverticulum and an epiblastic invagination.

Morgan and Tsuda (1894) locally pricked the gastrula surface and studied the evolution of the blastopore, from the onset of

gastrulation until blastopore closure. They described the original dorsal lip as travelling 120° vegetalward before it became a closed circle (Fig. 8). The ventral-most blastopore lip was considered a nearly fixed point. These observations too had led Morgan (1893) to criticize Roux's and Hertwig's above-mentioned concrescence theory, derived from His (1874).

H.V. Wilson (1900) tried an experimental study of blastopore formation in compressed eggs of the Anuran *Chorophilus*. He measured growth and movement of the blastopore slit, in an attempt to ascertain whether the dorsal lip overgrew the yolk cells. In pricked eggs, he obtained evidence for localized extraovate movements during gastrulation. Like Morgan and Tsuda (1894), at first he agreed that, if the ventral lip of the blastopore remained stationary, as Roux and Hertwig and Morgan believed, the dorsal lip travelled 120° vegetalward over the yolk, providing the material out of which the neural plate differentiated, according to the Roux's and Morgan's theory (Fig. 7). But in a second article, Wilson (1901) no longer maintained that the ventral lip remained stationary, and he stated that it overgrew vegetal yolk cells too. Therefore, the anterior part of the neural plate was considered as deriving from "tissue situated in front of the original position of the dorsal lip, and the [longer] posterior part [was] derived from tissue produced by expanding ectoderm during the closure of the blastopore". This conclusion, though it partly corrected the earlier erroneous view, was still far from what we know now.

In the Anurans, using Vogt's vital staining techniques, Pasteels (1943) much later demonstrated that blastopore fate was actually different according to the Amphibian models. In *Xenopus* and *Discoglossus*, blastopore transforms into the definitive anus, without being transiently occluded. In *Rana*, *Bufo* and *Hyla*, the blastopore is occluded and the definitive anus is a secondary formation.

### Is there a primitive streak in Amphibians?

In the preceding section, we have mentioned authors who believed that a primitive streak formed in Amphibians (Goette, 1875, Schultze, 1888 ; Houssay, 1890). However, at variance with
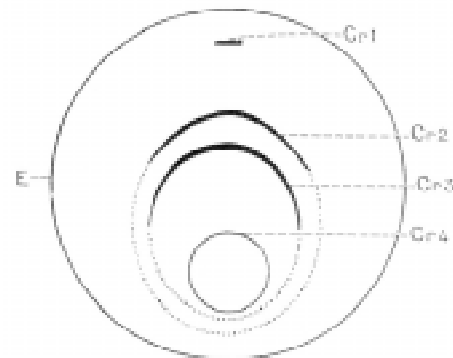


**Fig. 8. Schematic interpretation of blastopore closure in Amphibians, on a vegetal hemisphere view** *(adapted from Morgan and Tsuda, 1894). Cr1, 2, 3, 4: successive positions of the upper blastopore lip during gastrulation. Dotted lines indicate virtual positions of lateral and ventral parts of the blastopore. E, egg equator.*
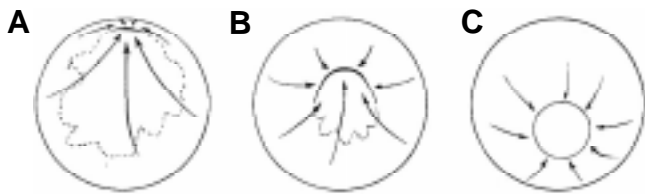
**Fig. 9. Morphogenetic movements at three successive stages of gastrulation** *(adapted from Kopsch, 1895b).* **(A)** *Endoderm cell movements towards the newly formed blastopore slit.* **(B)** *Convergence movements towards half-circled blastopore.* **(C)** *Later epibolic convergent movements at yolk-plug stage.*

the alleged formation of primitive streak by tissue concrescence from both sides in the bird embryo, there were different definitions of the Amphibian primitive streak. According to Johnson (1884), the primitive streak in the crested newt was present at gastrulation, along the neural plate, indicated by the "primitive groove". Later on, it persisted above the blastopore, evolving into the definitive anus : the description given permits assimilating this posterior "primitive streak" with tail bud tissues, into which the "medullary canal" gradually differentiates backwards.

As discussed by Robinson and Assheton (1891), the concrescence theory implied that the primitive streak should form in front of the residual blastopore. On the other hand, they explained how various authors had different opinions about the origin of streak formation. Robinson and Assheton themselves came to the view that, in the frog, the homologue of the chick primitive streak was to be found "in the whole of the blastopore lip, whether fused or not" : it subsequently gave rise to both dorsal and ventral halves of the internal tissues of the tail, thus being placed into what is nowadays named the tail bud.

The primitive streak concept was progressively abandoned during the next few decades, but it was still used by French embryologist Paul Wintrebert, who described its formation in the Anuran *Discoglossus* and never changed his views about it (Wintrebert, 1932). Using Vogt's vital staining techniques, he nevertheless opposed Vogt's results, which pointed to a lack of primitive streak in the formation of axial organs (Vogt, 1929).

Looking for a primitive streak in Amphibians was an attempt to establish closer homologies between chick and frog embryos. Similarly, earlier authors used the word *hypoblast* in both cases, hypoblast being later named *endoderm* in Amphibians, as in Invertebrates. Curiously, the presence of a true hypoblast in *Xenopus* has recently been questioned, following use of gene expression techniques : the initial expression of *crescent*, a *Wnt* antagonist, in the *Xenopus* blastula, allowed Pera and De Robertis (2000) to identify a deep endodermal region near the blastocoele in the dorsal blastopore lip and in the anterior mesendoderm. This region might be homologous to the chick anterior hypoblast, which expresses *crescent* too. In *Xenopus*, *crescent* is also expressed during gastrulation in the dorsal blastopore lip and the anterior mesendoderm, then in the prechordal plate.

### First evidence for morphogenetic movements (Kopsch, 1895)

The conflicting interpretations of gastrulation processes were all based on the examination of histological sections that appeared to be very similar in different species of the same animal group (Anurans or Urodeles). As emphasized by Morgan (1897), precon-

ceived ideas played a major role for several authors, but others seem to have been objective observers. All of them undoubtedly suffered from serious technical limitations. Few things could be more difficult than imagining dynamical changes affecting cell layers that had been subjected to fixative media. At the same time, experimental embryology was starting, due to pioneering work by Roux and his followers, studying "developmental mechanics". However, it must be emphasized that the new breakthroughs were obtained with incomplete descriptive morphological data, which could lead to misinterpretations of the experimental results.

A new approach to gastrulation processes was initiated by Kopsch (1895a, b), who presented his first results at a meeting of the Society of German Naturalists in Berlin, in February 1895.

Another presentation occurred in the same year at the meeting of the Anatomical Society in Basel. Kopsch had taken serial time-lapse photographs of precise superficial cell groups in frog and axolotl gastrulae, with an inverted microscope. The exposure time was 20-30 min, which allowed cell movements to be detected by blurred images, while motionless cell groups remained sharp. Cell nuclei were visible, allowing precise observations. Kopsch was thus able to detect convergence movements of ectoderm and dorsal lip cells towards the blastopore, as well as those of invaginating macromeres (Fig. 9). Kopsch's drawings clearly evoke those that were to be produced by Vogt and Holtfreter, 30 to 50 years later.

Following his observations, Kopsch discarded both theories of bilateral ectodermal epiboly over the macromeres (Roux, Schultze) and of delamination (Lwoff and others). He also concluded, although cells originally placed on both sides were indeed converging towards the median line, that the embryo was not formed by uniting two separate right and left halves : this was an additional refutation of His's concrescence theory (1874), supported by Roux and by Hertwig (1892). The discussion still remained open and experiments were performed to settle this point, for example by Hamecher (1905), who tried to decide where cranial and caudal growth areas were placed in relation to the blastopore margin.

The displacement of the blastopore itself from the dorsal to the ventral side of the vegetal hemisphere was clearly seen. The incipient blastopore in *Rana* was situated 25° under the equator and its complete migration towards the vegetal pole was estimated at 75°, whereas Roux, who believed that the blastopore first appeared at the egg equator, thought that the entire migration was much more extensive, about 170°. That corresponded to his above-mentioned view on dorsal organization in the vegetal hemisphere (Fig. 7). Kopsch's findings also opposed Schultze's opinion, that dorsal lip movement was only apparent.

In a subsequent article, Kopsch (1900) developed his observations on the problem of embryonic axes as related to early cleavage stages in the frog embryo.

We shall not comment on the first theories that arose in the 19th century about determination of axes (Born, Pflüger, Schultze, Roux). Kopsch observed a rotation of the gastrula relative to the initial vertical axis during development and schematized his findings with an interpretation of dorsal macromere fate during gastrulation (Fig. 10). He concluded that there was no relationship between the second cleavage plane and the determination of cranial and caudal parts of the embryo. The cell material that should have been cranial according to Roux was located partly in the dorsal neural plate and the archenteron roof, whereas the presumptive caudal material (accord-

ing to Roux) formed caudal and ventral parts of the embryo. Kopsch believed that both dorsoventral and craniocaudal axes were determined only at the end of gastrulation.

Roux (1902) analyzed and criticized Kopsch's results, arguing on experimental data obtained by himself and other authors. He still maintained that the dorsal part of the embryo was located on the vegetal hemisphere of the initial blastula and was formed by concrescence of bilateral material, cephalic and caudal respectively. Kopsch had dismissed Roux's statement that the second cleavage plane separated anterior (cephalic) and posterior (caudal) embryonic halves, the first cleavage having defined the plane of bilateral symmetry.

We must stress that Kopsch was the first author who brought direct evidence for complex morphogenetic movements during gastrulation. His results were widely known and commented on. Nevertheless, we shall see that some authors still believed in splitting phenomena during gastrulation and refuted invagination. However, a few others pricked the gastrula surface to obtain experimental markers, in order to obtain information on cell movements. Thus, in Chicago, Eycleshymer (1898) used a segment of hair maintained with forceps to make very small punctures on selected areas of gastrulae from *Ambystoma* and two species of the Anurans *Bufo* and *Acris*. The small exovates moved during gastrulation and neurulation and it was demonstrated that animal pole marks were incorporated into the head region, while exovates above the blastopore marked the posterior region. Invagination, evident all around the blastopore, was more important on the dorsal side. The author concluded that "the greater portion of the embryo arises in the darker hemisphere by differentiation *in situ* and not by concrescence".

Nevertheless, leading embryologists did not yet change their contrary views.

### Studies on gastrulation at the beginning of the 20<sup>th</sup> century

The preceding results should have brought major changes in the interpretation of gastrulation processes and of embryo formation, but they were still far from persuading prominent members of the embryological establishment that they had made faulty assumptions.

Keibel (1901) wrote a detailed account on Vertebrate gastrulation, including 12 pages of references. He proposed three definitions of gastrulation that arose from the bulk of the authors :

1. Gastrulation is a process during which the cells that will form the intestinal lining find their way into the interior of the embryo;
2. Gastrulation is the process during which the material for notochord and mesoderm find its way into the interior of the embryo ;
3. Gastrulation is the process by which the material for endoderm, mesoderm and notochord finds its way into the interior of the embryo.

Keibel chose the third definition for Vertebrates, pointing out that the first one was only appropriate for Invertebrates and it tacitly assumed the primary importance of invagination, according to Haeckel's views. However, he emphasized the fundamental distinction of two primitive cell layers, external and internal, regardless of how they were obtained, in a diploblastic embryo.

Going back to this discussion, Hubrecht (1905) pointed out that fundamental gastrulation proceeded by delamination in Vertebrates, with the exception of *Amphioxus,* where invagination occurs, to obtain a two-layered embryo. The leader of Dutch
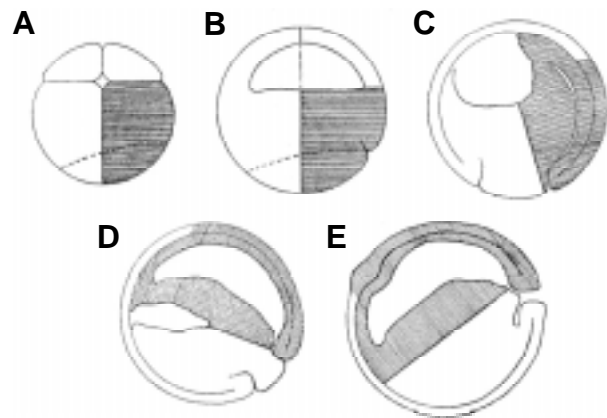


**Fig. 10. Fate of dorsal vegetal blastomere material** *(hatched) from 8-cell stage* **(A)** *on, during successive gastrula stages* **(B to E)***, drawn according to sagittal sections. The original vertical axis, imposed by gravity is kept on all figures, which makes rotation of the embryo during gastrulation obvious. The presumptive dorsal side (on the right in A and B) moves upwards during gastrulation. In (A) and (B), the broken line indicates the limit of brown and white areas (adapted from Kopsch, 1900).*

embryologists believed that it was not justified to include the whole process of notochord formation (= notogenesis) in the gastrulation phenomenon, nor that of the coelome and the somites. He then chose among gastrulation processes only those that had a counterpart in Invertebrates. Commenting on this article, Keibel (1905) again agreed with Hubrecht on most of his points. Both of them first proposed a scheme of gastrulation in two phases, but Keibel rejected the second phase (notochord and mesoderm formation) as being peculiar to Vertebrates and therefore proposed to retain the first phase as general to both Invertebrates and Vertebrates.

Both authors thus considered phylogenetic considerations to be most important in defining embryonic developmental stages, as Haeckel did, but they corrected his definitions and rejected the strict homology between gastrulation and invagination. Indeed, no invagination could be observed in bird gastrulation.

At the same time, on Kopsch's suggestion, Adler (1901) studied toad gastrulation on classical histological sections, of which he published a few microphotographs of good quality. He observed continuity of the endodermal cell layer on the dorsal side, as earlier emphasized by Schultze (1888). He did not find any evidence for an origin of mesoderm from ectoderm, endoderm or coelomic diverticula, once more opposing Haeckel's and Hertwig's theory, and he concluded that mesoderm derived from a group of yolk cells already present at the blastula stage and that migrate to their definitive location during gastrulation. Ectodermal, mesodermal and endodermal precursor cells should already be separated at that stage, ready for later differentiation processes. The early gastrula already had three germ layers.

Controversial objections were still to be put forward for more than two decades, until Vogt published his famous experiments with vital staining (1923-1929) and put an end to the main debates on invagination and delamination.

The most surprising opposition to a dynamic interpretation of gastrulation came from the great Belgian embryologist Albert Brachet (1903), who still believed that an accurate histological analysis could lead to a good understanding of gastrulation.
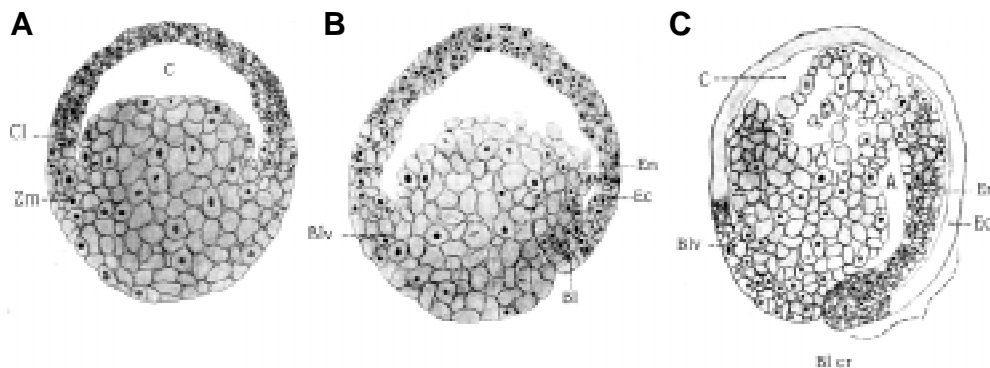
**Fig. 11. Sagittal sections through three early stages of Axolotl development** *(adapted from Brachet, 1921).* **(A)** *Late blastula.* **(B)** *Early gastrula.* **(C)** *More advanced gastrula. A, archenteron; Bl, blastopore cranial (or dorsal) lip; Blcr, extended cranial lip; Blv, blastopore virtual caudal lip (ventral marginal zone); C, blastocoele; Cl, 'gastrular cleavage' (Brachet's cleft); Ec, ectoderm; En, endoderm; Zm, marginal zone.*

Brachet's long article dealt with axolotl and brown frog (*Rana temporaria*) development and included a detailed study of gastrulation. In Brachet's view, it actually started at the blastula stage, prior to the onset of blastopore formation, by a "gastrular cleavage" between the blastocoele floor and the ectodermal wall (Fig. 11A) : segregating cell movements in the blastocoele floor were thus implied. Separation of ectoderm from endoderm first occurred with "gastrular cleavage". Onset of blastopore formation was paradoxically considered as the starting point of blastopore closure, but not of gastrulation, for which "gastrular cleavage" was an essential step. The corresponding advanced blastula stage was named "gastrulation prophase". This interpretation shows how difficult it may be to clearly separate developmental steps. Of course, we consider the onset of gastrulation from an external view only, much more precise and practicable. According to Brachet, blastopore formation was only the beginning of endoderm invagination, since he opposed the invagination concept for ectodermal and marginal zone cells. Brachet had seen Kopsch's photographs and agreed with some of Kopsch's conclusions, but he nevertheless rejected involution of external cell layers at the blastopore lip level. Epiboly movements extending the blastopore dorsal lip towards the vegetal pole were considered as causing external cell layers to glide on the invaginating endoderm. Archenteron cavity formed by a splitting phenomenon inside endoderm only : its dorsal roof, now known as chordoblast, was considered endodermal as well. In this respect, as emphasized by Brachet himself, these views were the same as those earlier expressed by Schwink (1888). The blastopore lips were not involuting and had no special activity, only representing the moving front of epiboly movements. The entire mesoderm was considered to originate from endoderm cells by delamination processes. Notochord and mesoderm shared a common origin in the blastopore region, but notochord did not form from a common dorsal plate.

In his article, Brachet otherwise analyzed the numerous contributions that had been devoted to the topic and which he discussed accurately. He later summarized his views in the first edition of his embryological treatise (Brachet, 1921). A second edition was prepared from 1930 on, but the author unfortunately died in 1931, having rewritten a few chapters only. His collaborators Albert Dalcq and Pol Gérard continued the revision work and their analysis of Amphibian gastrulation incorporated the detailed investigations made by Vogt a few years earlier.

Meanwhile, a few articles still appeared on Amphibian gastrulation, which progressively led to recognition of Kopsch's ideas. Working on yolk-rich eggs of *Desmognathus*, Hilton (1909) clearly described a small-celled archenteron invaginating into a large-celled endodermal substrate.

Working on the Dipneust *Lepidosiren*, Kerr (1901) had shown great similarities between gastrulation processes in this pulmonate fish and those of Urodele Amphibians, and also with those of the lamprey. He concluded that gastrulation occurred by a true process of invagination and that notochord and mesoderm rudiments were at first continuous across the mid-line. These conclusions, which were obtained through study of abundant material, agreed with Kopsch's interpretation of gastrulation. Brachet (1903) opposed this interpretation and argued that initial archenteron formation occurred as a cleavage process in all these cases.

Further progress in analyzing morphogenetic movements in Amphibians was realized by Goodale (1911, 1912). For the first time, he used Nile Blue sulphate as a dye marker for vital staining of the cell yolk granules. Spots around the equator of a *Spelerpes* blastula were shown to elongate from the equator to the blastopore edge along meridians of the gastrula. From the examination of sections, he concluded that the endodermal yolk cells and the cells of the marginal zone were "in a condition of active movement. Neither kind moves the other passively, but each is actively moving in coordination with the other to its proper place". He rejected increase in cell division number or increased growth of local areas as a cause of invagination in other areas. The study of histological sections was completed with an experimental study on living embryos. The dye mark was applied with a needle to the surface of an egg whose perivitelline fluid had been removed. The first experiments, performed in 1905 and 1906, allowed Goodale to define complex convergent movements towards the blastopore lips. He discussed the concept of convergence as related or opposed to that of concrescence, used by other investigators. He refused the former views expressed by His, Hertwig, Roux and others, considering that the embryo was formed from two separate halves by apposition. Morgan (1894) had accepted the concrescence theory, "not by apposition but by fusion from before backward at the dorsal lip of the blastopore". In Goodale's experiments, two marks placed one in the dorsal lip and the other in the lateral lip both reached the tail. Goodale found confirmation of his conclusions in a previous study by King (1901), who used exovate marks in *Bufo* and substantiated the view of the formation of the posterior part of the embryo by convergence.

Marks obtained by pricking the embryonic surface were still used by Dutch embryologist Delsman (1916a, b). At odds with Hubrecht's prevailing opinion, he wrote : "Nothing pleads for and everything against concrescence" in the frog embryo. In his opin-

ion, in *Rana fusca (=R. temporaria),*blastopore closure took place exactly at the vegetal pole, whereas it occurred more on the dorsal side in *Rana esculenta.*

Such contributions to descriptive embryology were becoming more and more obsolete with the breakthroughs that were brought by experimental microsurgical methods, under such prominent leaders as Spemann in Germany and Harrison in the USA. But these methods still suffered from very approximate mapping knowledge, still the subject of controversial debates. The precise limits of neural tissue, even more the exact position of head and trunk in the embryo, were not mapped on the gastrula surface. This situation did not prevent Spemann and Mangold from performing their landmark experiments on the organizing centre during the period 1921-1924. But a precise analysis of gastrulation processes was not yet available : Vogt's observations following vital staining of small areas were still in progress and were extensively published in 1925 or 1929 only.

Kerr (1919), although he was convinced of both the reality of involution movements and the superficial extension of the dorsal lip of the blastopore, did not reject the existence of delamination processes in gastrulation. He even attributed to them a key role in internal mesoderm formation from primitive endoderm.

### Bottle cells: the case for cell movement

The first author who carefully described the presence of elongated cells whose narrow apex lined the bottom of the blastoporal pit was Italian embryologist and cytologist Angelo Ruffini (1907a, b), although "bottle cells" already had been seen and drawn, without comments, or described without drawings by a few authors (Ruffini,1925). Early investigators of gastrular histological structures ignored them.

Ruffini (1907a) examined *Rana, Bufo* and *Triturus* embryos and once more discussed gastrulation events. He drew a schematical section of an Anuran blastula on which he localized various organ-forming areas. He was right in ascribing mesodermal value to the internal marginal zone. In later gastrulation stages, after involution, dorsal mesoderm of ectodermal origin was added to this annular anlage. Arguing about previously mentioned gastrula definitions by Brachet (1903), Hubrecht (1905) and Keibel (1905), Ruffini concluded that a distinction between gastrulation itself on the one hand, giving rise to a two-layered embryo only, and notogenesis on the other hand, during which mesoderm and notochord formed, was not relevant to Amphibian gastrulation. It was not possible to coin a general definition of gastrulation that applied to all Metazoans, "from Hydra to man", as wanted by Hubrecht (1905).

In his second article, Ruffini (1907b) concentrated on newt gastrulation, which he studied on several thousands of histological sections. His article was illustrated with several microphotographs, unfortunately of poor quality. He described definitive endoderm as originating in an external area of the blastula, characterized by club-shaped cells at blastopore slit formation. These cells reversibly flattened and multiplied during the emergence of the archenteron wall, but the forward archenteron extremity was still formed of such club-shaped cells, that were later named "bottle cells" by English-speaking authors, or also designed as "Ruffini's cells" (Fig. 12).

Ruffini considered that endodermal bottle cells played two fundamental functions, expressed in their shape : 1) a moving role, driving archenteron elongation through neighboring yolk cells,
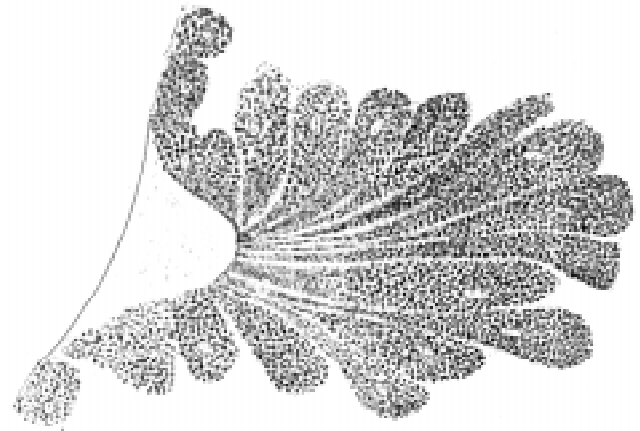


**Fig. 12. Bottle cells at the onset of blastopore formation in a *Triturus* gastrula** *(from a microphotograph by Ruffini, 1907, 1925).*

towards the blastocoele cavity ; 2) a secretory exocrine role, which could exert a chemotactic influence on mesodermal cells, attracted by mucous secretions. The second point was more hypothetical, since mesoderm cells were seen migrating in front of archenteron invagination and not only following it.

Ruffini deserves credit for having emphasized the importance of physicochemical explanations in embryonic development and not only of mechanical forces, as did some earlier investigators (His, 1874 ;Rhumbler, 1899, 1902). He still devoted an article to the relationships between "amoeboid movement" and secretion. Both functions were themselves related to organ formation and body shaping (Ruffini, 1908). Histochemical reactions in bottle cells allowed him to discuss their secretory role. On the other hand, the general occurrence of bottle cells in morphogenesis was emphasized by their presence, not only in the blastopore and the archenteron, but also in folds on both sides of the medullary groove during neural tube formation. Ruffini rejected the hypothesis of active rising of the neural folds during neurulation, believing that it corresponded, on the contrary, to oblique bilateral ingression movements, inducing the raising of medullary folds and their subsequent fusion on the mid- line.

Postponing evaluation of Ruffini's later contributions, we shall now examine finding on bottle cells made by earlier authors. In 1884, another Italian embryologist, Giuseppe Bellonci, had published a long descriptive article on blastopore formation and the primitive streak in Vertebrates. He paid particular attention to axolotl development and carefully described gastrulation, where invagination played a major role. Three processes were clearly distinguished : 1)involution of surface cells and consecutive internal doubling of the external layer ("primitive ectoderm"); 2) direct penetration of the involuted cells inside the blastopore slit; 3) very probably, amoeboid movements of the so-called "protoplasmic cells" as opposed to yolk cells, i.e., presumptive endoderm and mesoderm cells. The latter point referred to Stricker's observations on amoeboid movements displayed by frog embryonic cells *in vitro* (Stricker, 1864b). Mesoderm cells were less adherent to each other and moved in an interstitial liquid medium.

Bellonci already rejected Hertwig's theory on the endodermal origin of mesoderm. He clearly drew bottle cells in the archenteron cavity, but he did not make special comments on their individual
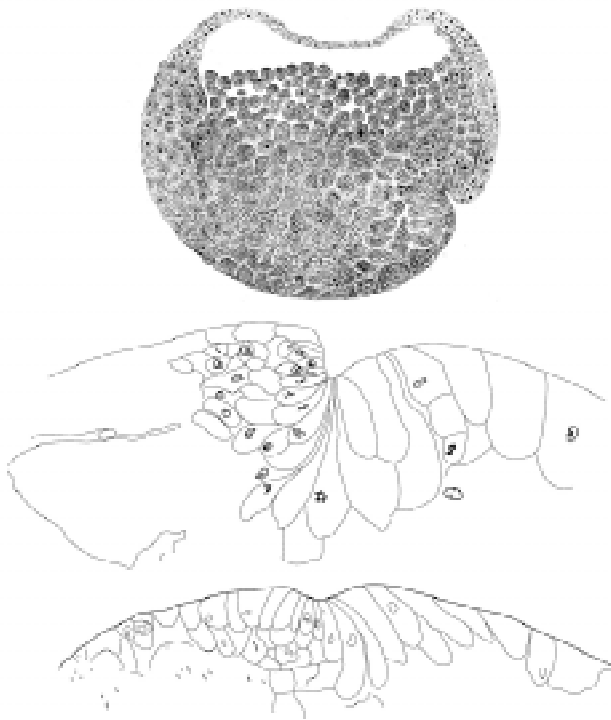
**Fig. 13. Bottle cells in the *Alytes* gastrula** *(adapted from Seeman, 1907). The top figure is a sagittal section through an early gastrula, where club-shaped cells are visible in the blastopore pit. On the dorsal side (right), migration of mesodermal cells on the inner surface of the blastocoele roof has already started. On the opposite side, Brachet's cleft is clearly visible. The two lower line-drawings show sections of an early blastopore, with different shapes of nascent bottle cells.*

shape and functions, as Ruffini did later on, considering only the overall behavior of invaginating endoderm cells that formed an epithelial sheet behind their moving front. Bellonci also studied primitive streak characters in avian blastoderm, and ended his article with comparative considerations about Vertebrates. Altogether, his studies seem to have been more relevant than those of many of his followers. Ruffini cited Bellonci's article and later mentioned his drawings of "club-shaped cells" (Ruffini, 1925).

Houssay (1890) also drew bottle cells in axolotl gastrulae, recognized the pigment accumulation at their apex, but did not comment on their shape, being convinced that archenteron formation proceeded by splitting and not by invagination.

Morgan (1894) did not draw bottle cells, but clearly described them : "The cells along the blastopore crescent pull in from the surface, leaving only their small pigmented ends for a time exposed. This process continues, the elongated cells pull in beneath the surface and a narrow space is formed between the yolk cells". For the first time, a correlation was thus suggested between the shape of bottle cells and an active movement inwards.

At the archenteron opening, Rhumbler (1899) described in Urodeles elongated radiating cells whose external apex was heavily pigmented. This was supposed to be correlated with active, cell autonomous immigration movements. Later on, he studied gastrula invagination as a phenomenon of developmental me-

chanics, on *Rana* embryos from Roux's preparations, still displaying bottle cells. Rhumbler analyzed multiple possibilities linked to the chemical composition of blastocoele fluid, cytotropism, different cell affinities for internal medium and external cell membranes, and the role of compression forces (Rhumbler, 1902). He attributed to Goette (1875) the first explanation of endoderm cell deformation by ectodermal cell compression. Rhumbler's mechanical interpretations of development were strongly criticized by Ruffini (1925), although the German author had developed views on active endoderm cell migration.

King (1902) carefully examined sections through the blastopore opening and her cell drawings show bottle cells nicely outlined, but she did not make any remarks about them. Bottle cells were also represented but were not commented upon in *Lepidosiren* gastrula (Kerr, 1901).

In the same year as Ruffini, Seeman (1907) clearly described club-shaped or bottle cells in blastopore formation, in *Alytes* (Fig. 13). Independently of Ruffini, he suggested that this cell shape could be the morphological expression of a nascent movement, but he also considered that it might originate from compression exerted by expanding ectoderm. He was aware of Morgan's interpretation and of the presence of similar cells in gastrulae from other animal species, but he did not examine the complete evolution of cell shape changes (Ruffini, 1925).

Therefore, if these cells were later named "Ruffini's cells", it is not because Ruffini was the first author to mention them, but because he clearly ascribed a leading role to them in gastrulation and morphogenetic movements, described the reversibility of their shape changes and stressed that they played an important physiological role, being endowed with secretory activity. A few years later, he entrusted Laura Marchetti with the task of looking for as many aspects as possible of the occurrence of bottle cells in the development of *Bufo* organs (Marchetti, 1914). She obtained spectacular pictures of cement gland formation from ectoderm, following Ruffini's observations on other invaginating systems (olfactory and otic vesicles, stomodaeum and proctodaeum).

Ruffini (1925) later published a big treatise on morphological and physiological aspects of embryology, entitled *Fisiogenia*. The book displayed of course numerous figures of bottle cells in various invaginating developmental structures and very long comments on them. Exhaustive reference lists were given at the end of each chapter. The author frequently used a curious style, sometimes lyrical, sometimes polemical, frequently enthusiastic, but always clear. He particularly combatted Hertwig's coelome theory, which had already been criticized by many embryologists, but was still extant due to the great influence of this German author and of his everlasting text-books. Ruffini emphasized, among other relevant topics, the simultaneous presence of the three germ layers at the very beginning of gastrulation, tracing back their origin to the blastula. In this respect, he was very close to modern views. Mesoderm cells were rightly believed to be located around the blastopore (peristomal origin). However, Ruffini was soon attacked by Vogt (1929), who showed from his vital staining experiments that other conclusions of Ruffini's analysis, notably on notochord and mesoderm segregation, were contradicted in Amphibians. Ruffini's statements on germ layer formation were considered obsolete. Nevertheless, we shall see that Vogt himself could be mistaken in describing the origin of Anuran mesoderm. The last section of Vogt's landmark article (Vogt, 1929, pp. 659-697) dealt

with a historical recapitulation and a discussion of general theories on early development (axis determination, theory of concrescence, coelome theory, blastopore theory). We shall now evoke this celebrated author, whose achievements put an end to old controversies and gave birth to our modern knowledge on morphogenetic movements.

### Analysis of gastrulation and morphogenetic movements by means of vital staining: Vogt (1929) and his followers

Having designed an original method of vital staining by means of agarose blocks carefully placed on the surface of the embryo, Vogt (1925, 1929) obtained indisputable evidence for complex movements involving groups of gastrular cells. His two landmark articles are widely known and illustrations selected from them are reproduced in every text-book of developmental biology. Summarizing this work would be at the same time impossible and beyond the scope of this review. We shall only indicate the main steps that led Vogt to this survey and discuss a few points.

Vogt's first contribution to newt embryogenesis appeared when he was still a student (Vogt, 1909). The second (Vogt, 1913) dealt with cell movement and cell degeneration during gastrulation and more specifically with the behavior of isolated embryonic cells. Vogt was not the first author to work on this topic and he gave a historical account of earlier contributions that apperared during the second half of the 19<sup>th</sup> century, since the first observations by Remak (1851). After World War I, Vogt resumed his work on cell movements *in situ*, having used *en masse* vital staining to mark specific grafts on unstained embryos. Vogt later explained (1925) that he was unaware of Goodale's studies on vital staining with Nile Blue (1911, 1912) as well as those of Detwiler (1917) when he started his own experiments. Having mixed unstained cells with cells stained by neutral red, he observed that the dye did not move from cell to cell. Goodale had used fragments of stained dry agar, placed on the embryo. Vogt used a more convenient method, the stained agar being first hydrated. In 1922, Vogt published his first results on involution and stretching of the blastopore dorsal lip. This allowed him to define the territories normally giving rise to notochord anlage and to somites. Their movements were autonomous, independent of cell growth, and only involved reorganization (Vogt, 1922a). This work was completed by experiments on exogastrulation (Vogt, 1922b) that clearly led to rejection of the concrescence theory and of a two-layered gastrula stage in Amphibians. On this occasion, Kopsch expressed his satisfaction at seeing his earlier works (1895a, b) confirmed. This was followed by another preliminary communication (Vogt, 1923a) in which the origin of the entire mesoderm was clearly ascribed to the blastula marginal zone, without any participation of endoderm, the coelome theory being thus definitely rejected. In another communication (Vogt, 1923b), the first comprehensive drawings of mark movements were published. Vogt insisted on the advantages of his vital staining technique, which did not perturb organogenesis, in opposition to microsurgical operations (stitching, microburns, extirpations).

The details of his vital staining techniques, their aims and advantages were published in a first landmark article (Vogt, 1925). Vogt waited four years before issuing the second detailed article dealing with the interpretation of gastrulation and mesoderm formation (Vogt, 1929). In the period between them, other articles were published, dealing with several experimental topics using Amphibian eggs. Results obtained by one of Vogt's colleagues
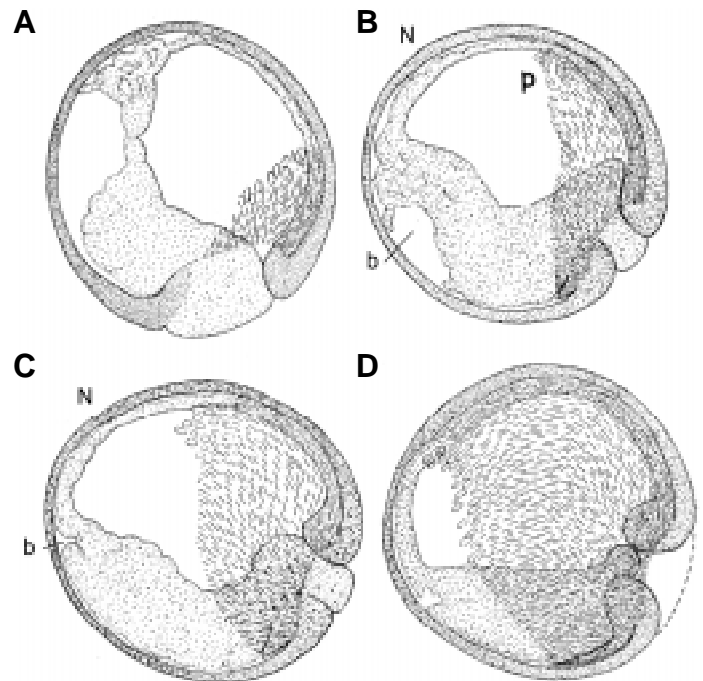


**Fig. 14. Diagrammatic sagittal sections through *Pleurodeles* gastrulae**, *with mesodermal mantle superimposed (dashed). b, blastocoele; N, anterior part of the neural plate; p, dorsal edge of the mesodermal mantle whose migration can be followed from early* **(A)** *to advanced* **(C)** *and late* **(D)** *gastrula stages (adapted from Vogt, 1929).*

using his technique, were also published (Goerttler, 1925, 1927). They concerned presumptive neural plate and morphogenetic movements during neurulation. All these studies were made in parallel with founding experiments on gastrulation and the organizer in Spemann's laboratory. Whereas the older authors mainly studied gastrulation in Anurans, the Urodele egg was most advantageous for experimental procedures and had been selected by Spemann at the beginning of the 20<sup>th</sup> century (Beetschen, 1996). It was selected too for vital staining because of its clearer pigmentation.

Vogt's 1929 article has been constantly cited ever since its appearance. It was illustrated with 95 photographs, drawings and schemes. It provided a complete analysis of Amphibian gastrulation, mostly on *Triturus*, *Pleurodeles*, *Ambystoma* as Urodeles, and *Bombinator* as Anuran. The fundamental morphogenetic movements were described and classified : invagination, stretching, convergence, divergence, epiboly. Notochord and somitic mesoderm areas were shown to converge towards blastopore lips, where their rolling up and involution were demonstrated. This confirmed results from transplantation experiments by Mangold (1923), which also excluded the possibility that mesoderm arose by delamination or evagination of endoderm and highlighted the role of invagination and involution phenomena during gastrulation. The progressive immigration of the mesodermal mantle from behind towards dorso-lateral and anterior regions of the gastrula was especially apparent (Fig. 14).

This does not mean that all of Vogt's interpretations were definitive, since several details, especially those concerning the Anuran fate map, were later corrected. But Vogt's work was the
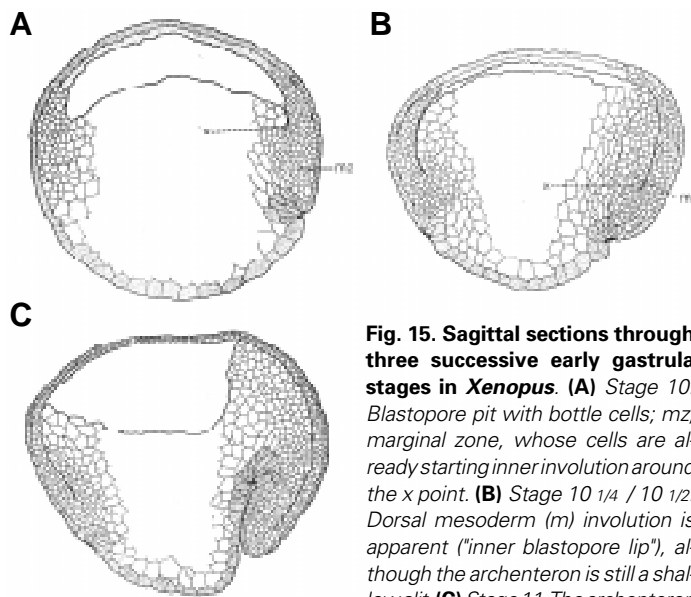
**A**



**B**



**C**



**Fig. 15. Sagittal sections through three successive early gastrula stages in *Xenopus*. (A)** *Stage 10. Blastopore pit with bottle cells; mz, marginal zone, whose cells are already starting inner involution around the x point.* **(B)** *Stage 10 1/4 / 10 1/2. Dorsal mesoderm (m) involution is apparent ("inner blastopore lip"), although the archenteron is still a shallow slit.* **(C)** *Stage 11. The archenteron is formed, with bottle cells at its front end; the ventral blastopore slit is forming. The externalmost cell layer (ectodermal or endodermal) has been carefully drawn all around the embryo. Harrison's original stages have been renumbered according to the later normal developmental table by Nieuwkoop and Faber (1967). (Adapted from Nieuwkoop and Florschütz, 1950).*

cornerstone on which all subsequent contributions could lean. Our current representation of gastrulation events dates back to Vogt's analysis, immediately used by experimental embryologists, who relied on his detailed fate maps.

Vogt unfortunately died from cancer in 1941, aged 53. A complete list of his publications can be found in an obituary article by Spemann (1941).

Following in his footsteps, several embryologists soon applied Vogt's technique to the study of other Anuran species or of organogenesis in the same Urodele species. The maps of prospective organ-forming areas were thus completed or corrected (Wintrebert, 1932 ; Pasteels, 1936, 1942). The mesoderm-forming area in Anurans was later revealed not to be superficial, as it is in Urodeles, but to be covered with an external epithelial monolayer (Dettlaff, 1936) : this derivative of the blastula's bilayered "primitive ectoderm" and its endodermal vegetal counterpart, which characterize the Anuran gastrula (Figs. 5, 15), is the source of differences between Anuran and Urodele gastrulation. In Anurans, it involutes around the blastoporal lip together with the inner layer cells to form the epithelial lining of the archenteron, thus displaying its endodermal prospective value. Ventral to the notochord, the external cell layer forms the hypochordal plate (Vogt), which is lacking in Urodeles, where the "primary ectoderm" and the chordamesoderm are monolayered (Dettlaff, 1983, 1993). Older authors already had described this external cell layer in Anurans (Stricker, 1862 ; Van Bambeke, 1870 ; Schultze, 1888).

## II. An overview of modern advances in gastrulation studies

### Xenopus *gastrulation*

Investigations in *Xenopus* gastrulation started with a developmental table and a histological analysis of successive gastrula stages (Fig. 15) by Nieuwkoop and Florschütz (1950). This work described a full internal localization of marginal zone material in the early gastrula, a hypothesis that had to be confirmed by experimental procedures. On the other hand, immigration of internal cells appeared to begin prior to blastopore formation, corresponding to Brachet's older concept of "gastrular cleavage".

Criticizing Vogt's fate maps, LØvtrup (1965, 1966, 1975) then believed that the *Xenopus* situation might be extended to all Anurans and Urodeles too, where he suspected that vital dye marks in the marginal zone might have been internalized by diffusion, leading to an erroneous conclusion about their presence in a superficial cell layer.

The first systematic study of fate maps and morphogenetic movements in *Xenopus* was performed by Keller (1975, 1976). No mesoderm could be mapped in the superficial cell layer of the early gastrula. The suprablastoporal endoderm invaginated as a continuous layer to form the archenteron endodermal roof. Morphogenetic movements of prospective mesoderm were investigated in detail. Notochord derived from the dorsal deep layer of the marginal zone. Strong dorsal convergence of mesoderm and ectoderm was associated with divergence in the ventral region.

These articles were the first of a long series of publications by Keller and his collaborators, dealing with cellular aspects of gastrulation, not only in *Xenopus* but in other Anurans as well. Cinemicrographic techniques, SEM studies, surface measurement and analysis of mophological cell changes, isolation of explants and defect experiments in gastrulae, etc., were largely used (Keller 1978, 1980, 1984, 1986 ; Keller *et al.*, 1985 ; Keller and Danilchik, 1988 ; Keller and Tibbets 1989 ; Keller and Jansa, 1992 ; Keller *et al.*, 1991, 1992 ; Wilson and Keller, 1991 ; Shih and Keller, 1992 a, b, c).

A process of radial intercalation was first described (Keller, 1978), by means of which two cell layers intermingle their individual cells to form one cell layer only, covered with flattened superficial cells of a third epithelial layer. This contributes to increase gastrula surface during epiboly.

A second process of mediolateral intercalation was then analyzed. In the marginal zone, deep cells of the axial mesoderm become intercalated between one another to form longer but narrower arrays during convergence and extension movements. This process was also described in surface epithelium (Keller and Tibbets, 1989). Gastrulation without a blastocoele roof was later analyzed (Keller and Jansa, 1992). It was shown also that the marked outer epithelium of the dorsal marginal zone (DMZ) had organizer properties when grafted in the ventral side of a recipient gastrula. It contributed cells to deep mesodermal tissues and developed itself into mesoderm when grafted into the deep region. Prospective endoderm epithelial cells thus might influence mesoderm cell behavior during normal development (Shih and Keller, 1992 c).

Another gastrulation movement has been described recently in *Xenopus* by Winklbauer and Schürfeld (1999), who named it "vegetal rotation". Here the prospective endoderm actively moves inwards into the blastocoele, leading to an expansion of the blastocoele floor and a turning around of the inner marginal zone, prior to mesoderm involution. This movement initiates the formation of Brachet's cleft. These results imply an active global participation of the vegetal cell mass in emboly phenomena rather than a passive role of the yolk cells.

A descriptive and experimental study of tension-dependent cell movements in *Xenopus* gastrula ectoderm during epiboly was

recently published by Beloussov *et al*. (2000), a further contribution from these authors' laboratory to the study of mechanical forces occurring in embryo formation.

We should also mention a series of investigations dealing with the clonal origin of different parts of the early *Xenopus* gastrula, from early cleavage stages onward (16-32 cells). Clonal studies were actually initiated 30 years ago by Nakamura and Kishiyama (1971). A step forward concerned the clonal origin of neural anlagen (Hirose and Jacobson, 1979 ; Jacobson and Hirose, 1981). Dale and Slack (1987), Moody (1987) and Takasaki (1987) published fate maps for the 32-cell stage. A similar clonal analysis at the 32-cell stage was performed in the frog *Rana pipiens* (Saint-Jeannet and Dawid, 1994). Much more detailed cell fate maps during *Xenopus* gastrulation were published by Bauer *et al*. (1994), after injection of fluorescent dextran into each of the blastomeres at 16-or 32-cell stage. This study was particularly aimed at defining the clonal origin of the Spemann and Mangold organizer, which was ascribed to dorsal blastomere B1 at the 32-cell stage. Relative movements of cell clones during blastula and early gastrula stages were also clearly revealed.

Comprehensive fate maps compiled from different authors' results over the years have recently been subject to certain revision. Keller (1991) modified his own fate map by accomodating part of the somitic mesoderm into the presumptive ventral part of the marginal zone mesoderm. Recently, Lane and Smith (1999) proposed a more radical change based on the origin of primitive blood cells : they found that a significant number of tadpole blood cells originate from dorsal equatorial blastomere C1, which also corresponds to the organizer. They hypothesized a double annular structure for the whole marginal zone, the lower vegetal ring representing ventral mesoderm and the upper one dorsal mesoderm. Starting from this point, Lane and Sheets (2000) used lineage labeling and development of embryonic isolates to propose a complete revision of primary axis determination in *Xenopus* and amphibian gastrula. According to these authors, the classical dorsoventral axis should in fact be considered to correspond to the anteroposterior axis. The axis orientation of the final tadpole is curiously reminiscent of Roux's old theory in which the head was superimposed on to the grey crescent and presumptive notochord area (Fig. 7), which marked the anterior end and not the definitive dorsal side of the embryo. A detailed discussion of this rejuvenated hypothesis would be outside the scope of the present essay. We only wish to point out that a close correspondence between egg and gastrula axes on the one hand and those of the advanced embryo on the other hand might be only theoretical in an Amphibian : migrations and morphogenetic movements displace original cell positions, intermingle, juxtapose or separate cells whose initial fate is rightly considered presumptive. The classical interpretation of dorsoventral polarity (Hertwig, 1882 ; Morgan, 1897 ; Vogt, 1929) relied on notochord position in the fate map. The definitive notochord lies on the same side as its presumptive material, whose movements only affect its extension along the prime meridian passing through the upper lip of the blastopore. It is finally characterized as a dorsal and not as an anterior structure, since it does not extend into the entire head. The dorsoventral polarity of marginal zone presumptive mesoderm in an early gastrula might be different from the definitive polarity that is acquired in the late gastrula, when somitic dorsal mesoderm flanks the notochord anlage.

Recently, gastrulation was investigated in two other Anurans, *Ceratophrys* and *Hymenochirus,* in Keller's laboratory. In the South American toad *Ceratophrys*, which is not closely related to *Xenopus*, Purcell and Keller (1993) observed a new phenomenon, ingression, by which some prospective notochord, somite and posterior mesoderm cells leave the surface epithelium of the archenteron to join the mesodermal anlagen. This occurs in three zones of the archenteron roof and around the blastopore, and shows that a proportion of mesoderm cells actually have a superficial origin in this Anuran species. In *Hymenochirus*, a close relative of *Xenopus* (*Pipidae*), it was also shown that dorsal mesoderm partially originates from superficial cells of the involuting marginal zone epithelium. Groups of cells from the archenteron roof join the somite and the notochord anlagen, in which they are incorporated (Minsuk and Keller, 1996). The authors describe the specific behavior of these cells as "relamination". It thus appears that *Xenopus* might be an exception among all Anurans so far investigated, having no longer retained prospective superficial mesoderm. Two years earlier, in the frog *Rana pipiens*, Delarue *et al*. (1994) had demonstrated that surface cells also contribute to mesoderm.

Reading these numerous articles shows how complex morphogenetic movements are and how we are faced with new problems at the cell and molecular levels. Keller himself very appropriately summarized a series of questions about them, in an article in which he paid homage to Holtfreter's pioneering work on gastrulation (Keller, 1996). We shall examine later the contribution of Keller's group to the role of *Xenopus* bottle cells in gastrulation, and of other authors as well in other modern aspects of gastrulation analysis, when Urodele and Anuran specificities will be compared in subsequent paragraphs.

### Urodele gastrulation

Though Urodele models had been central to Vogt's analysis of gastrulation, modern studies paid less attention to them than to *Xenopus*. Nevertheless, several contributions deserve mention, due to initial differences in surface cell layers between Urodeles and Anurans, inducing differences in invagination and involution mechanics (Dettlaff, 1993).

Lundmark (1986) still used vital dye marks, completed with fluorescent dextran tracer, in the axolotl, *Ambystoma mexicanum*. The superficial notochord anlage was studied during involution and completion of the dorsal roof of the archenteron. Endodermal tissue was shown to become immediatly adjacent to the notochordal material, confirming Vogt's view that the endoderm is brought into apposition with the lateral edges of notochord. This result challenged the older erroneous view according to which endodermal dorsal free edges migrated dorsally across chordomesoderm to meet in the midline (Fig. 1). Bilateral ingression of superficial mesodermal cells makes the apposition of endodermal and notochordal cells possible. The migration of mesodermal cells will be considered later.

Shi *et al*. (1987) performed grafting experiments to study the behavior of the DMZ in *Pleurodeles*. In non-involuted DMZ, radial intercalation leads to the appearance of a single layer of cells. Animal pole cells also form a single layer. DMZ transplanted into the animal pole region showed no autonomous extension. A 180° anteroposterior rotation of the DMZ did not interfere with its involution, but removal of the blastocoele roof at blastula stage, leaving the DMZ on the cut

edge, blocked it. At the early gastrula stage, involution could still proceed partially. These last results are at variance with those obtained in *Xenopus* by Keller *et al.* (1985) and by Keller and Jansa (1992), who showed that removing the blastocoele roof did not prevent convergent extension and DMZ involution to occur, followed by blastopore closure.

There are clearly significant differences between Anuran and Urodele gastrulation mechanisms. There is no autonomous convergent extension of the axial mesoderm in *Pleurodeles* according to Shi *et al.* (1987). According to Keller and Jansa (1992), convergence and extension mechanisms in the axial mesoderm are relatively weak but occur later in Urodeles. The clonal analysis of the derivatives of each blastomere at the 32-cell stage of the *Pleurodeles* embryo was also performed (Delarue and Boucaut, 1992) and followed by a more detailed fate map of marginal zone mesoderm cells, using double-labelled grafts and injection of individual cells with a fluorescent tracer. The respective contribution of superficial and deep cells was established (Delarue *et al.*, 1992). These studies brought evidence for mixing and rearrangement of cells from different clones during early development.

Using double labelling (lysinated-rhodamine dextran and $^{125}$I), Delarue *et al.* (1996) compared a Urodele (*Pleurodeles*) and an Anuran (*Rana*) once again. They placed small defined grafts of the DMZ orthotopically on unlabelled host embryos and compared migrations of superficial and deep cells along the anteroposterior axis. A much greater mixing beween superficial and deep cells occurred in *Pleurodeles* than in *Rana*. Deep cells were the first to involute and these then divided into two subpopulations, anterior and more posterior, whose migrating behavior was different.

More recently, Suzuki *et al.* (1997) observed extensive regulation capacities of the japanese newt (*Cynops*) gastrula, after removal of the dorsal-vegetal quarter, including prospective notochord, blastopore and endoderm. Lateral areas of the marginal zone and extended involution led to formation of a well-differentiated notochord and of a dorsal axis in operated embryos.

### Analysis of mesoderm cell migration and of gastrulation movements

This fundamental approach has been already presented in the preceding paragraphs. To study the control of gastrulation, it was necessary to experiment not only on whole embryos or on separate parts of these embryos, but also *in vitro* on isolated cells, subjected to various environmental conditions. It was necessary too to induce gastrulation movements in cells which would not normally display these phenomena: this was recently obtained by use of mesoderm-inducing factors (activin, fibroblast growth factor, bone morphogenetic protein-4) acting on ectodermal isolated caps (review in Howard and Smith, 1993). It thus became possible to tackle such important questions as : when do cells know that they should gastrulate, how and by which factors is their behavior modified ? Precise descriptions of these various cell behaviors were of course prerequisite for these studies, but constituted only a primary step (review in Keller and Winklbauer, 1992).

It had long been suspected that inner cells from the marginal zone display autonomous mobility capacities (Stricker, 1864 b), thereby explaining how cells originating from the blastocoele floor adhere to the inner wall of the blastocoele roof at the beginning of gastrulation. Vogt (1913) had observed isolated gastrula cells *in vitro* that formed pseudopodia. He did not cite Stricker, but mentioned several authors

of the last quarter of the 19$^{th}$ century who had envisaged cell migration as a factor to be taken into account in gastrulation.

As a former student in Spemann's laboratory, Johannes Holtfreter can be considered as providing a link between the foundations of classical experimental embryology and new developmental approaches during the second half of the 20$^{th}$ century. His achievements have been very clearly set out by Hamburger (1996 a, b). Having devised his well-known standard solution in the early 1930s, Holtfreter later studied the differentiation potencies of isolated parts of Urodele and Anuran gastrulae (Holtfreter 1938 a, b). He then introduced the concept of "tissue affinity" as a means of explaining embryonic morphogenesis. He had combined *in vitro* fragments of two or three germ layers of gastrulae. When fractions of ectoderm, mesoderm and endoderm were present, the ectoderm formed an outer envelope, the endoderm an inner vesicle and the mesoderm scattered cells between the epidermoid envelope and the endodermal vesicle (Holtfreter, 1939). These pioneering experiments were followed by studies involving different combinations of endoderm, mesoderm and ectoderm explants taken from gastrulae (Holtfreter, 1943, 1944). Marginal zone explants were shown to form an archenteron pouch when associated with a substrate of ectoderm plus endoderm. Formation of pseudo-blastoporal pits were frequently observed in such reassociation experiments.

During the last 25 years, new technical improvements allowed further insights into gastrula cell behavior. Both Anuran and Urodele models have contributed to our knowledge of mesoderm cell migration and of its molecular bases, involving extracellular matrix (ECM). Following electron microscopic evidence for changes in the synthesis of extracellular components at gastrulation (Kosher and Searls, 1973 ; Johnson, 1977 a, b), Nakatsuji *et al.* (1982) observed a network of extracellular fibrils, covering the inner surface of the ectodermal layer during gastrulation in *Ambystoma*. In both *Ambystoma* and *Pleurodeles*, Boucaut and Darribère (1983) showed by immunocytochemistry that the ECM network, appearing at the end of blastula stage, contained fibronectin (FN). In the japanese newt, Nakatsuji *et al.* (1985 a) demonstrated the presence of laminin in the same gastrula ECM network. FN was simultaneously detected as a substratum for cell migration in *Xenopus* gastrula (Nakatsuji *et al.*, 1985 b). Boucaut *et al.* (1984, 1985) had already shown that injecting antibodies to FN into the blastocoele blocked involution movements and inhibited gastrulation in *Pleurodeles*. Ultrastructural studies showed mesoderm cells attached to the ECM network during normal gastrulation, but not in blocked gastrulae. These studies were completed with *in vitro* investigations, showing that DMZ cells can migrate and spread on specific ECM-conditioned substrata (Shi *et al.*, 1989). In maternal-effect mutant gastrulae of *Pleurodeles*, in which invagination and involution movements are strongly impeded, Darribère *et al.* (1991) found an absence of fibrillar ECM. The inner blastocoele roof only displayed sparse granular ECM, which again pointed to the role of normal fibrillar ECM in emboly cell movements.

Nakatsuji and his collaborators studied *Xenopus* gastrula cells extensively (review in Nakatsuji and Hashimoto, 1991), whereas Boucaut and his collaborators mainly worked on Urodeles (review in Boucaut *et al.*, 1991). Other authors too contributed to studies on *Xenopus* gastrula cells in relation to ECM (Winklbauer, 1990 ; Winklbauer *et al.* 1991 ; Winklbauer and Selchow, 1992 ; De Simone, 1991). Winklbauer *et al.* (1992) insisted on the fact that cell aggregates, but not single cells, were able to follow guidance cues present in the ECM fibrils of the blastocoele roof. Aggregated cells appeared

unipolar and moved continuously, while isolated cells were bi- or multipolar and changed their direction of movement frequently. Cohesion of the mesoderm cell mass thus appears an essential factor of mesoderm cell migration in *Xenopus* gastrula. Since the fibrillar network appears less organized in *Xenopus* than in Urodeles, its guiding role in *Xenopus* has been controversial. Competitive RGD peptides, which act as ligands on FN cell receptors, did not arrest gastrulation movements in *Xenopus* (Smith *et al.*, 1990) but they did so in *Pleurodeles*. Nevertheless, antibodies to FN inhibit gastrulation in *Xenopus* (Howard *et al.*, 1992) as well as in *Pleurodeles*.

In *Rana*, at variance with *Xenopus*, it was experimentally demonstrated that adhesion of mesoderm cells to the FN-rich fibrillar ECM was required for normal gastrulation, as it is in Urodeles (Johnson *et al.*, 1993).

A further analysis of the situation in *Xenopus* was presented by Winklbauer and Keller (1996). According to these authors, an interface must exist between migrating mesoderm cells and the blastocoele roof cells to allow for migration : FN is not essential for this cell adhesion to occur, but interaction with FN is necessary for the formation of lamelliform cytoplasmic protrusions in mesoderm cells. The specific role of FN in migration would be to control cell protrusive activity, which is essential for migration. Nevertheless, involution and gastrulation still proceed in the presence of FN inhibitors. Movement of mesoderm cells thus should not depend on FN only, but on other factors still to be identified.

 Cell motility was further investigated by Wacker *et al.* (1998), who distinguished three types of motile cells in the dorsal half of the gastrula. Migratory behavior appeared to characterize anterior mesoderm and endoderm. Activin induced migratory activity in blastocoele roof cells, and so did bFGF, with different responses. Activin alone induced the expression of *goosecoid* and *Mix.1* in treated ectoderm caps. Injection of the mRNAs coding for these transcription factors showed that *gsc* had no effect when expressed alone, but it acted in synergy with *Mix.1* in the control of cell adhesion. *Mix.1* is normally expressed throughout the vegetal hemisphere at the early gastrula stage (Rosa, 1989).

It is known that the *Brachyury* gene in Vertebrates is necessary for posterior mesoderm formation and notochord differentiation. In *Xenopus*, Xbra acts as a transcriptional activator and is essential for convergent extension movements during gastrulation, but its function is not a prerequisite for adhesion and migration of mesoderm cells on FN (Coulon and Smith, 1999). Among the target genes of Xbra appears *Xwnt 11*, which is expressed in the marginal zone. *Xwnt 11* function is necessary for convergent extension movements but not for specification of mesoderm (Tada and Smith, 2000). It might act via activation of *Xdsh* (*Xenopus Dishevelled*), which has also been implicated in convergent extension (Sokol, 1996). Evidence for a crucial role of the Wnt signalling pathways in the control of morphogenetic movements has been accumulating during the past years.

Wnts are a large family of secreted cysteine-rich proteins, implicated in a wide variety of biological processes, including basic developmental mechanisms (Wodarz and Nusse, 1998). Their receptors are known as Frizzled, a family of seven-pass transmembrane proteins. Among the corresponding genes, *Xfz7* has been characterized recently as a maternal gene (Sumanas *et al.*, 2000). Depletion of Xfz7 results in loss of dorsoanterior structures and markers of dorsal mesoderm. Djiane *et al.* (2000) established that it interacts with Xwnt 11 and that its overexpression delays mesoder-

mal involution and affects convergent extension movements. Xdsh, downstream of Frizzled, synergizes with Xfz7. Similarly, overexpression of a mutant *Xdsh* inhibits convergent extension (Wallingford *et al.*, 2000). Xdsh signaling might control cell polarity decisions during convergent extension of the DMZ.

During the last few years, molecular investigations have also involved characterization of ECM receptors, and highlighted the importance of cell-surface integrins that attach to FN, laminin, etc. and play a key role in cell-cell and cell-matrix interactions (Skalski *et al.*, 1998 ; Darribère *et al.*, 2000). More detailed molecular analyses of the role of FN in cell migration have been performed too (Darribère and Schwarzbauer, 2000). There is thus now an impressive mass of cell and molecular biology data concerning both mesoderm cell migration and overall cell movements, of which we have only given a partial overview. This is an open and promising research field, since the role of specific gene activity in these phenomena remains poorly understood.

### Bottle cells: origin and functions

Following the enthusiastic description of bottle (or flask, or club-shaped) cells by Ruffini (1925), nearly 20 years elapsed before Holtfreter (1943) brought an experimental contribution to the analysis of their behavior in gastrulation. His work was performed on Urodeles (*Triturus*, *Ambystoma*). It was linked with the theory of "surface coat", described as an elastic, viscous, extracellular material providing cohesion to superficial cells. From *in vivo* gastrula dissections, Holtfreter confirmed several of Rhumbler's (1902) and Ruffini's (1925) observations on bottle cell characteristics. He considered that dorsal bottle cells at the time of blastopore formation were endodermal, but that ventral bottle cell fate was mesodermal. *In vitro* experiments showed that isolated bottle cells cultured on glass in an alkaline medium can spread and migrate on the substratum by their basal ends, whereas their apical ends remain non-adhesive. Explanted on an endodermal substrate, bottle cells form a kind of archenteron pit (Holtfreter, 1944). They were suggested to pull surrounding cells along with them, due to the cohesion attributed to the "surface coat".

Later, ultrastructure of bottle cells was carefully investigated by Baker (1965), who proposed a scheme of elongation and retraction during invagination, based on a theory of expanding and contracting fibrillar dense material that was seen accumulating in the apical, constricted end of flask cells. It was later suggested that the dense layer corresponded to badly preserved microfilaments (Keller, 1981). Perry and Waddington (1966) had also observed detailed ultrastructures.

A true experimental analysis of bottle cell role was performed by Keller (1981) in *Xenopus*. Keller distinguished involution and invagination processes and characterized gastrulation as an involution of the deep and superficial cell layers of the marginal zone. Deep cells were proposed to play an essential, active role. In contrast, the superficial layer including the bottle cells would be passively moved inside because of its attachment to underlying deep cells. According to this idea, bottle cells have no essential role, because involution can proceed after they have been extirpated at the beginning of gastrulation (stage 10). Their function would be to deepen the archenteron as a result of their shape changes : as already pointed out by Ruffini (1907 b), they spread and flatten and thus increase the archenteron epithelium surface. Keller thus dismissed the earlier views that bottle cells were the most active migrating cells during gastrulation.

Considering the bilayered structure of marginal zone in Anurans and the pecularities of *Xenopus*, it is not sure that similar investigations in Urodele embryos would give identical results. Using an array of techniques, including mechanical simulation, Hardin and Keller (1988) reinvestigated *Xenopus* bottle cells, taking into account these prospective differences. Their results clarified and somewhat modified Keller's earlier conclusions : apical constriction of bottle cells may indeed initiate involution of the deep marginal zone, whose extension and convergence properties would come into play later. The surrounding tissues act mechanically to modulate the contractile properties of bottle cells, which are uniform in all directions when the cells are isolated. Respreading behavior was considered to be an intrinsic property of bottle cells, since it occurs in cultured isolated cells. The authors noted differences between *Xenopus* and *Ambystoma* bottle cells : in the latter case, lateral and ventral bottle cells are mesodermal ; they ingress and invade the deep regions, losing their initial epithelial character. In *Xenopus*, bottle cells are all endodermal and remain in an epithelium sheet.

Keller later reconsidered the whole problem, starting from Holtfreter's famous contributions (1943, 1944). He still stressed the many points that remained to be elucidated : "what bottle cells do, how they do it, in a biomechanical sense, and how much diversification of bottle cell fonction exists within one species, as well as between species. " (Keller, 1996, p. 261). The molecular basis of these diverse functions in terms of cell adhesion, regulation of motility and shape changes was also evoked.

In this respect, recent work by Kurth and Hausen (2000) might be considered as a pioneering attempt to characterize genes involved in the determination of bottle cell formation. These authors induced ectopic bottle cells in the animal region by injecting mRNAs coding for members of the TGFβ family or VegT. It was known that activin-like TGFβ signals can elicit mesodermal properties in isolated animal caps, and that these caps are competent to form ectopic invaginations resembling blastopore lips. In the injected embryos, ectopic bottle cells are very similar to dorsal blastoporal cells. *Goosecoid* is expressed in the deep cells underlying ectopic bottle cells, as it is in the normal DMZ, but not in bottle cells themselves. The authors conclude that bottle cell formation is closely linked to mesodermal patterning in the subepithelial tissues and propose a general scheme of genetic and growth factor interactions in the process. Nevertheless, generalizing these statements might be premature. Ectopic bottle cells can form in a purely ectodermal environment in Urodeles. A maternal-effect mutant of *Pleurodeles waltl*, whose gastrular invagination was impeded in correlation with ECM anomalies (Darribère *et al.*, 1991), displayed an animal hemisphere which became furrowed and pitted, due to disturbance of epiboly. Pits and furrows were lined with bottle cells whose ultrastructure was investigated too (Bluemink and Beetschen, 1981). Bottle cell ingression and ectoderm anomalies were reversible, since a normal neural plate eventually formed when gastrula invagination and involution had proceeded further. In this case, dorsal bottle cells became epithelial again and the animal region always remained fully ectodermal. The determination of bottle cell formation thus still remains an open question.

### *Are epibolic and inward movements independent of each other?*

Spreading of ectoderm cells toward the vegetal hemisphere had long been recognized by early investigators. We mentioned that Brachet (1903) interpreted Kopsch's experiments (1895 a, b) as a demonstration of epibolic movements covering the prospective endodermal area, but that he refused to admit the occurrence of active invagination processes. Contrary to mesoderm cell movements, ectoderm spreading has been little investigated so far. The possible relationships between epiboly and invagination or involution movements have nevertheless long been questioned. At the end of the 19th century, several authors considered that epiboly was an active movement pushing endodermal cells inside, whereas others believed that invagination was pulling ectodermal surface cells.

Since then, we have obtained some experimental evidence for a relative independence between these two categories of cell movement. In *Xenopus*, Keller and Jansa (1992) removed the blastocoele roof and observed the occurrence of subsequent gastrulation phenomena. In certain experiments, the non-involuting marginal zone (NIMZ) was removed alone or together with the animal cap (AC). It was shown that removing AC and NIMZ prevented neither involution of the remaining marginal zone nor blastopore closure, even when the operation was performed at stage 9 (blastula), prior to the onset of involution. Inner mesoderm cells accumulated as a thick clump, being deprived of their normal substrate for migration. Convergence and extension of notochordal and somitic mesoderm occurred independently.

Another type of disjunction between epibolic and inward movements was observed in *Pleurodeles* gastrulae. Lithium treatment of 32-cell stage embryos is known to provide a maximal enhancement of dorsoanterior structures in *Xenopus* : gastrulae are dorsalized and dorsoventral polarity is practically abolished (Kao and Elinson, 1988). In *Pleurodeles*, in which gastrulation proceeds slower than in *Xenopus*, epiboly movements were much accelerated in Li-treated gastrulae, covering the vegetal pole region. But archenteron invagination was constantly retarded and commenced with a delay of several hours compared with control gastrulae. Dorsalization of the entire marginal zone was confirmed by the ability of all its sectors to behave as Spemann-Mangold organizer (Shi *et al.*, 1990). This confirmed that an experimental separation is possible between epibolic and inward cell movements, which can occur independently and successively.

Quite recently, a new gastrulation-controlling molecule has been characterized in *Xenopus*, corresponding to maternal *Xoom* transcripts in the oocyte (Hasegawa *et al.*, 1999). Experiments with antisense *Xoom* RNA produced gastrulation defects which did not affect mesodermal marker genes. Convergent extension of mesodermal cells occurred normally, but the epibolic spreading movement of ectoderm was strongly affected (Hasegawa and Kinashita, 2000). Xoom is a membrane-associated protein and anti-Xoom antibodies caused gastrulation defects (Hasegawa *et al.,* 2001). It is suggested that Xoom protein may regulate the organization of actin cytoskeleton.

## Conclusions

The gastrula concept was born from Haeckel's studies more than 125 years ago, based on comparative embryology of various Metazoans. The gastrula stage was quickly recognized to be an essential developmental step, during which the germ layers that will give rise to definitive organ anlagen are segregated. However, the invaginating gastrula has no generalized character in Metazoans and was not recognized in Amphibians for a long time by

numerous authors. Controverse raged for decades. Fifty years were to pass before gastrular cell movements were made really visible by Vogt. The importance of involution and invagination movements, the reality of convergence and stretching or spreading of cell groups were then clearly established.

Following these essential discoveries, another 50 years were necessary to go further. During this period, Holtfreter's pioneering work on the living gastrula had no immediate consequences, although the concepts of tissue affinity and selective adhesion were landmarks in embryological research. The lack of appropriate techniques was a serious obstacle to solving the problems that were raised by Holtfreter.

During the last 20 years, cell and molecular biology techniques have been successfully used for this purpose. Nevertheless, in spite of a great deal of new data, we still do not know why cells begin to move at a given time and how the independent movements of various cell groups are coordinated to form the complex architecture of the definitive gastrula. A detailed account of the numerous unsolved problems of gastrulation has been published by Keller (1996). We already know that a great number of diverse molecules are involved in these phenomena and a few regulator genes have been characterized. The complexity of cell-cell and cell-matrix interactions and of signalling pathways is recognized but still largely unexplored.

For reasons of convenience, the starting point of gastrulation remains the external appearance of blastopore pit. On the other hand, it is known that internal cell movements already occur earlier than this, and the study of Brachet's cleft (1903) has been recently revived through an analysis of tissue separation during gastrulation (Wacker *et al.*, 2000).

Gastrulation is now considered as being consecutive to early inductive interactions that occur at blastula stages (mesodermal induction). The gastrula itself later harbours various inductions, of which the earliest recognized was neural induction. Many genes and growth factors are involved in these inductive interactions that prepare the neural and mesodermal patterns. Their study, which has been exploding during the last decade, constitutes an autonomous field of research. This is probably why the importance of the events leading to the setting up of the basic architecture that plays roles in exchanging morphogenetic signals has been comparatively neglected.

The Amphibian gastrula, whose original concept was immersed in Haeckel's phylogenetic theories at the end of the 19th century, has proved to be a crucial model for our understanding of embryonic development. It remains a modern research field that should reveal basic aspects of the organization of cell architecture. Old problems are still waiting for new solutions.

## Summary

The hypothetical *gastraea* concept, proposed by Haeckel (1874) to be an ancestral form common to all Metazoans, relied on the characterization of a *gastrula* stage in their embryonic development. The first steps that led to this characterization in Amphibian embryos fell into oblivion and deserve mention. Similarly, controversial debates about gastrula formation from the blastula, about simultaneous appearance of the three germ layers as opposed to a theoretical diploblastic embryo and about the occurrence of inward morphogenetic cell movements *versus* that of delamination processes, lasted for years. Following a half-century of polemic (1875-1925), Vogt's studies clearly and definitively established the reality and the complexity of morphogenetic movements, but this breakthrough long remained without further consequences. Holtfreter (1943,1944) illuminated unknown aspects of living gastrula cells and his observations helped to define many problems to be solved. During the second half of the 20th century, cell and molecular biology techniques, applied to the study of cell-cell and cell-matrix interactions, have brought new insights into the mechanisms of gastrula cell movements. Gene expression during these phenomena still remains an open question, as shown by a few recent studies: this situation strikingly contrasts with the many achievements that have been accomplished during the last decade in the analysis of induction phenomena during gastrulation.

KEY WORDS: *Gastrulation, morphogenetic movements, mesoderm, Amphibians*

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