suppressed chondrogenic and myogenic cells also synthesize peak I, II and III sulfated proteoglycans.

The experiments described above are incompatible with the repeated claims that presumptive chondroblasts, as well as nonchondrogenic cells, possess an active 'chondrogenic genotype', or that many kinds of embryonic cells turn off their chondrogenic program as they mature or 'stabilize'[3,6–13]. On the contrary, it is more likely that the synthetic program of the definitive chondroblasts is qualitatively distinct from that of its precursor, or mother cell, the presumptive chondroblast.

This stress of difference in synthetic options between presumptive chondroblasts and their daughters, the definitive chondroblasts, is consistent with the finding that only the latter synthesize the cartilage type II collagen chains[26]. Type I collagen is the only type of collagen chain synthesized in early limb bud cells, which consist largely of presumptive chondroblasts. Type II collagen chains are found in limb buds only after the definitive chondroblasts are observed microscopically. From these observations we suggest that a presumptive chondroblast itself does not have the option to transcribe those genes which regulate the synthesis of peak IV sulfated proteoglycans and type II collagen chains. On the other hand, following a quantal cell cycle, a given presumptive chondroblast yields daughter definitive chondroblasts which now have the option to transcribe the genes coding for the core proteins of peak IV sulfated proteoglycans and for type II collagen.

In the absence of an inductive interaction between somites and notochord or spinal cord either in vivo or in vitro, early somite cells fail to chondrify. In a succession of publications Lash and co-workers, and Strudel have variously interpreted this relationship as follows: 1. the notochord secretes a unique molecule that specifically induces undifferentiated somite cells to chondrify[5,27]; 2. somite cells 'spontaneously' transform into chondroblasts[14]; 3. collagen secreted and deposited around the notochord specifically transforms somite cells into chondroblasts[2]; 4. glycosaminoglycans synthesized and released by the notochord is the specific inducing molecule for chondrogenesis[29]; 5. committed chondrogenic cells migrate to the surface of the notochord and there chondrify[25]; and 6. local K ion concentrations are critical for such inductive activity[30].

In contrast to any of these explanations, Holtzer and co-workers[2,17,18,21,23] have suggested that the above experiments merely demonstrate that enrichment of the culture medium in which somites are growing will permit them to chondrify, providing that their past mitotic history has rendered them competent to do so. More specifically, the notochord or spinal cord permits endogenously programmed presumptive chondroblasts to undergo the quantal cell cycle that yields daughter definitive chondroblasts. The definitive chondroblasts are the only cells in, or outside of, the chondrogenic lineage with the option to transcribe and translate all of the proteins required for those sulfated proteoglycans that band under peak IV and that synthesize type II collagens chains. Simple exposure of presumptive chondroblasts or 'somite' cells or 'limb bud cells' to culture media enriched with exogenous glycosaminoglycans or collagen chains or to analogs of nicotinamide[13] cannot induce such cells to switch to the synthetic program characteristic of definitive chondroblasts. The most such exogenous molecules can do is to permit presumptive chondroblasts which divide in their presence to undergo the quantal cell cycle which yields definitive chondroblasts. As such, exogenous molecules play the relatively trivial role of 'trigger' rather than delivering information which instructs cells how to diversify.

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Yolk sac erythropoiesis

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The yolk-sac is an extraembryonic appendage with 2 functions: absorption of the yolk nutrients and genesis of blood cells during embryonic life. The blood cells produced are mainly erythrocytes. The erythropoietic function is especially striking in birds, because of its duration and the large number of cells formed. However, these 2 parameters can vary considerably among species, depending on the relative roles in haemopoiesis of other organs such as liver, spleen and bone marrow. In the chick, which has been the most studied species, the liver has no erythropoietic function and the yolk sac (YS) is active for the greater part of embryonic life[1].

Indeed, the YS is such a conspicuous blood forming organ in the chick that it has been ascribed in recent years a unique role in the establishment of the whole haemopoietic system. According to this view, the YS is the sole progenitor of all haemopoietic stem cells (SC)[2].

The descriptive and experimental data pertaining to the differentiation of the YS blood islands and the types of erythrocytes formed sequentially will be reviewed here. However, the main emphasis will be directed to a re-examination of the role of the YS in the ontogeny of the haemopoietic system. Personal experimental data bring new light on this problem: the evolution of the whole haemopoietic system has been analyzed in quail embryos, developing with a chick YS from a young stage[3,4]. In this new type of chimaera[5], cell movements are traced by means of Le Douarin's quail-chick marker method[6]: the prominent heterochromatin masses present in quail nuclei are identifiable in the maturing cells of the erythroid series and in all stages of the other haemopoietic lines (figure 5).

1. *Differentiation of the blood islands of Wolff and of the yolk sac circulation*

The wall of the YS is the extraembryonic splanchnopleure (splanchnic mesoderm + endoderm) (figure 1). It becomes individualized during the 3rd day of incubation, as the extraembryonic coelom extends centrifugally splitting the extraembryonic mesoderm into 2 layers. The term 'yolk sac erythropoiesis' encompasses red cell production by this individualized wall, and also earlier formation of erythrocytes in the area opaca of the expanding blastoderm before 2 days of incubation. The early events preceding the establishment of blood islands have been studied by explantation in organ cultures of fragments of the blastoderm[7–11]. Haemopoietic potentialities are already determined as early as the pre-primitive streak stage and are relatively uninfluenced by subsequent cell movements. Migration of precursor cells through the primitive streak occurs as a part of the gastrulation process. At the definitive streak stage, haemopoietic precursor cells are arranged in a horse-shoe shaped region postero-lateral to the area pellucida (figure 2).

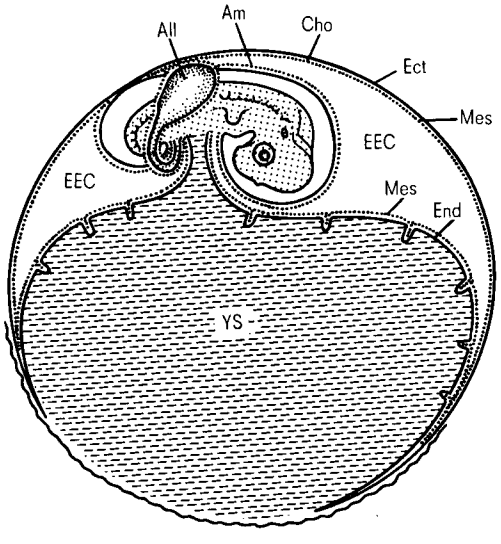

Fig. 1. Diagram of the relations of the chick embryo and its appendages on the 4th day of incubation. Redrawn after Hamilton[13]. All, allantois; Am, amnios; Cho, chorion; Ect, ectoderm; EEC, extraembryonic coelom; End, endoderm; Mes, mesoderm; YS, yolk sac.

Description of the differentiation of blood islands is based on studies by Dantschakoff[1], Sabin[12] and Hamilton[13].

The first blood islands become visible around the headfold stage (18–24 h of incubation) imparting a mottled aspect to the area opaca. Developing in the three-layered part of the opaque area, they appear first behind the embryo, then rapidly progress forward on each side of the embryo; their differentiation follows the expansion of the mesoderm. At first they are formed of tight clusters of basophilic cells. These will differentiate into endothelium and free hemoglobinized erythroblasts in another 10–14 h. Hemoglobin first becomes detectable cytochemically at the 6- to 8-somite stage around 36 h of incubation[11]. As the blood islands differentiate, the three-layered portion of the area opaca develops into what is known as the area vasculosa, which soon acquires a visible boundary, the marginal vein or sinus terminalis (figure 4). The two-layered peripheral portion of the opaque area is called the vitelline area. As the formation of blood islands spreads centripetally, they become a network of capillaries. Movement of blood begins at the stage of 16 somites, when complete circuits are opened in the capillary network, though the heart has already begun to contract sporadically at the 9-somite stage.

Miura and Wilt[14] have sought, in organ culture experiments, evidence for tissue interactions which might occur during early YS erythropoiesis. Ectomesoderm of the area vasculosa of the definitive primitive streak stage cultured alone displays a limited capacity to from blood islands. Endoderm exerts a positive influence across a filter which is believed to prevent cell contacts.

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Thus, although dispensable, endoderm enhances haemopoiesis in associated ectomesoderm. Rather than a specific tissue interaction, analogous to those demonstrated in several embryonic systems, the endoderm might exert a stimulating effect on the multiplications of stem cells by transmitting nutrients and possibly more specific factors from the yolk. Further research would appear useful to clarify this point.

Haemopoiesis in the YS is at its peak between the 10th and 14th or 15th days and then declines. It gradually comes to an end between the 18th and 20th days of incubation.

2. Erythroid cell populations

a) *Primitive erythrocytes: megalocytes.* They form a homogeneous population with unique features. Released in the circulation as immature cells, at the proerythroblast stage, they mature synchronously in the blood. They are the only circulating red cells until about 5 days of incubation[15]. They are large and spherical with a large nucleus. They synthesize 3 major hemoglobins (Hbs), the so-called E, P and M Hbs in Ingram's terminology (for a review, see Bruns and Ingram[16]) (figure 3).

b) *Definitive erythrocytes: normocytes.* They begin to appear in the circulation at 5 days of incubation and flow in at such a rate as to make up 50% of the total erythrocyte count around 8 days[16].

The first ones are released immature, as basophilic erythroblasts or polychromatophils. They are smaller than megalocytes, slightly elongated with round nuclei. Cultures of 'de-embryonated' blastoderms have demonstrated that the YS gives rise to normocytes, as well as to megalocytes[17].

As the age of the embryo increases, immature normocytes appear to be retained in the haemopoietic organs until the late polychromatophilic or reticulocyte stage. In older embryos, red cells are produced which are elliptical with oval nuclei. It is likely that several erythroid populations differing in morphological and biochemical criteria are produced, but they coexist so that it is impossible to obtain them pure[1,18–20].

Their existence is inferred from the evolution of Hbs (figure 3). These are characterized by 3 bands, 1 of which (band H) co-migrates with embryonic band E. Hb H is a transitory one which disappears 1 month after hatching. The 2 other bands are the adult Hbs A and D, which are present in particular proportions and slowly evolve towards the adult ratio. Whereas it has been ascertained that Hbs E, P and M are restricted to megalocytes[21], the relationship between the other Hbs and the different types of successive normocytes has not yet been determined. Neither is it known how many normocytic generations are derived from the YS.

3. Evaluation of the role of the yolk sac as haemopoietic stem cell progenitor in quail chick chimaeric germs

It was Maximov[22,23], who first proposed that all blood cells were derived from a common precursor, a stem cell arising from fixed mesenchymal cells. This monophyletic or unitarian theory was opposed by various others. No agreement was reached for many years, until experimental data could relay histological observation. In the last 15 or 20 years, ample experimental evidence has been acquired for the existence in the adult vertebrate bone marrow of a self-sustaining reserve of free-wandering stem cells.

In the embryo, pioneering work by Moore and coworkers, using the sex chromosomal difference of the chick as a marker, demonstrated traffic of cells between the various haemopoietic compartments. Their evidence was obtained from embryos parabiosed in ovo, according to Hasek's technique[24], or from irradiated embryos, injected with YS cell suspensions; in both experiments, the haemopoietic organs were chimaeric[2,25–29]. These authors inferred that SC do not differentiate in the haemopoietic organ rudiments, which have to be seeded by extrinsic SC. This hypothesis was confirmed by experiments carried out in our laboratory, using the quail chick marker system; the timing of colonisation of the lymphopoietic organs and bone marrow was carefully analyzed by interspecific grafting of rudiments[30,31].

The site of origin of SC during embryogenesis thus became the focal point. Considering that the YS is the first site of embryonic haemopoiesis, Moore and Owen put forward a hypothesis according to which some SC, formed in the early YS, do not enter the first generation erythropoietic compartment but divide to form further SC which colonize the intraembryonic organs via the circulation: '... the formation of stem cells from more primitive precursor cells, therefore, may be a developmental event unique to the early embryo ... The stem cell pool of the adult, which is also thought to

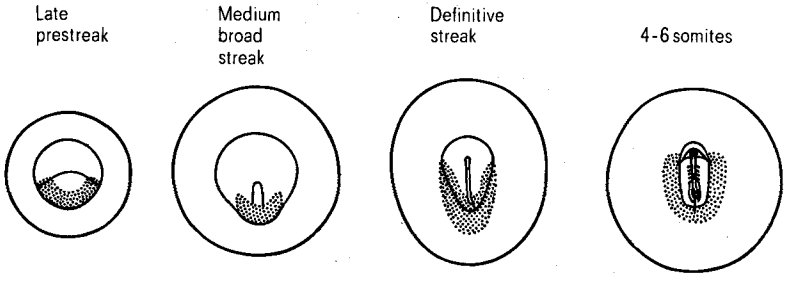

Fig. 2. Erythropoietic potentialities in the young blastoderm. Redrawn after Settle[9]. The stippled areas show the regions of potential haemopoiesis, which were identified by the capacity of isolated portions to form blood cells in vitro.

show self-replication, may in turn be derived from these embryonic sites'[2].

This appealing hypothesis has met with widespread acceptance and is usually considered as proved by experimental data. However, in the parabiosis system used by Moore and Owen, the YS of the 2 partners are present, as well as possible intraembryonic sources from the 2 parabionts. Thus, although chimaerism is clearly demonstrated, there is no proof that the exchanged cells arose from the YS.

We have challenged this interpretation on the basis of results obtained in chimaeras composed of quail embryos deprived of their own YS and grafted with a chick YS[3,4]. These are obtained by surgical grafting of a quail area pellucida on a chick area opaca at stages ranging between 8 and 14 somites (figure 4). Circulation is established after the operation. The chimaeras are sacrificed at various ages from 5 to 13 days of incubation. The development of their haemopoietic system is monitored by several parameters, which all reveal the existence of an intraembryonic site of formation of SC. The species of the cells (quail or chick) making up the haemopoietic organs is analyzed in Feulgen-Rossenbeck stained sections. The proportions of chick and quail erythrocytes in the blood is measured by an immune haemolysis technique using rabbit antibodies against either quail or chick red cells[4]. Immune haemolysis is also used to isolate chick erythrocytes (= yolk sac derived) or quail erythrocytes (= embryo derived) in order to study the electrophoretic patterns of their Hbs at different critical stages of development.

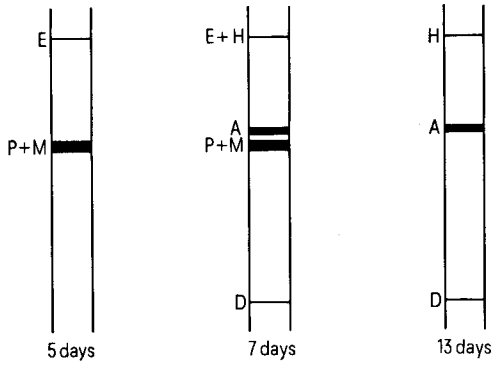

Fig. 3. Evolution of the Hb pattern in the developing chick embryo. The bands are resolved by polyacrylamide gel electrophoresis in the conditions defined by Bruns and Ingram[16].

The haemopoietic organs of the chimaeras are predominantly populated by quail haemopoietic cells (HC). Thymus and bursa of Fabricius, the primary lymphoid organs, contain only quail lymphocytes or lymphoblasts in more than 95% of the chimaeras. The spleen, which undergoes a phase of active erythropoiesis at 11–15 days, shows transitory and variable colonisation by chick HC at 11–12 days. The bone marrow, when beginning to differentiate in 12–13-day chimaeras, contains only quail HC[32].

Thus, overwhelming predominance of quail HC is the general rule in the intraembryonic organs of the chimaeras. The quail HC can only be derived from SC which were differentiated in the embryo proper. Moreover, in a fair proportion of cases, from 7 days on the YS blood islands of the chimaeras contain a mixture of quail and chick haemocytoblasts or erythroblasts (figure 5). Thus after a certain time of incubation the YS functions with SC from the embryo.

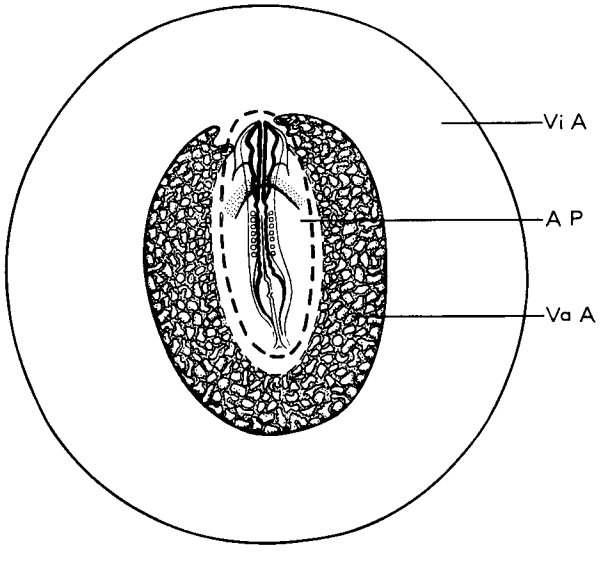

Fig. 4. Scheme of the grafting operation for producing quail embryo-chick YS chimaeras. The dotted line indicates the location of the seam between the 2 blastoderms. The central zone of the chick blastoderm has been removed and replaced by the corresponding quail region. AP, area pellucida; ViA, vitelline area; VaA, vascular area.

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The limited ability of the YS as a SC progenitor is further substantiated by the evolution of the blood composition (table).

Age (days)	Number of chimaeras	Mean value of the percentage of quail erythrocytes
5	9	< 5
6	31	6.3
7	26	6.95
8	14	10.46
9	12	16.55
11	13	17.8
12	19	26.2
13	25	43.32

The difference between day 5 and day 13 is highly significant. Until 5 days of incubation the YS appears as the main progenitor of erythropoietic SC; thereafter it is relayed by the embryo proper.

The source of SC in the embryo probably resides in the general mesenchyme; this is suggested by the presence in the vicinity of the dorsal aorta of haemopoietic foci which were repeatedly described at the turn of the century[18,22,33]. In the quail, 2 prominent symmetrical foci are regularly present at the level of the subcardinal veins in the 7–8-day embryo.

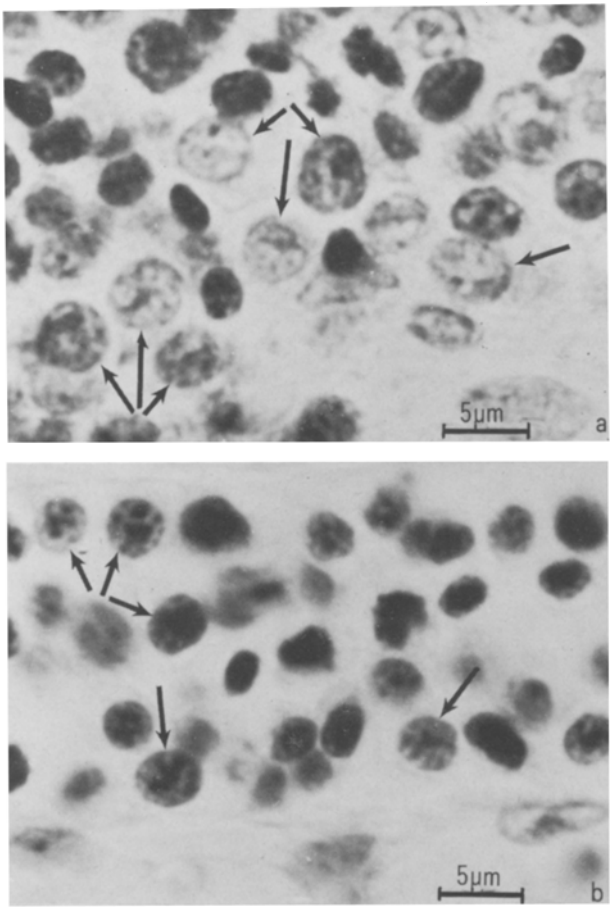

Fig. 5. YS blood islands of 2 'YS chimaeras'. Feulgen-Rossenbeck staining. *a* Erythropoietic cells are of chick type. The large clear nuclei are typical of chick haemocytoblasts (arrows). *b* Erythropoietic cells are of quail type. Haemocytoblast nuclei are smaller and contain a heavy heterochromatic mass (arrows).

Recently, experiments were performed in a chick-chick combination, following the same design. Area pellucida of one blastoderm was grafted onto area opaca of another, from the same inbred chicken strain. The sex chromosomal marker was used to trace the origin of cells in the thymus, bursa of Fabricius and bone marrow of 'sex-chimaeras' at 16–19 days of incubation. These organs were never found to contain cells of the opposite sex, which would have originated from the YS[34]. Thus the results in the intraspecific combinations confirm previous ones, excluding possible disturbance of SC homing by developmental difference in the interspecific chimaera.

Traffic of SC from the embryo towards the YS was also suggested by Samarut and Nigon[35]. These authors have studied the ontogenesis of the chick haemopoietic system by adapting to this species the classical mouse irradiation – reconstitution technique of Till and McCulloch[36]. They find that, on the 6th day of incubation, there are 3 to 4 times more CFU-Ms (colony forming units in marrow) in the blood than in the YS. Thus the evolution of the haemopoietic system in quail chick YS chimaeras clearly demonstrates the existence of 2 SC populations. One becomes functional early, gives rise to megalocytes and arises from the YS; the other becomes functional in a 2nd step, gives rise to normocytes and white blood cells and forms in the embryo proper. However, several data indicate some overlapping of the sites where these 2 SC populations differentiate, and also show that their potentialities are not radically different:

1. Chick erythrocytes, retrieved from 7-day chimaeras, contain normocytic and megalocytic Hbs. Thus the YS gives rise to erythrocytes of both generations. This fact, already established by Hagopian and Ingram[17], in the 'de-embryonated' blastoderm experiment, is also manifest in the quail chick system.
2. The small, but significant, percentage of quail erythrocytes present in chimaeras at 5 days of incubation are megalocytes, as shown by their Hb content. Several controls show that these megalocytes are produced by the area pellucida and not by 'contaminating' quail area opaca introduced at grafting[37].
3. Quail head-fold stage area opaca associated with a chick thymus rudiment gives rise to quail lymphoblasts[38].

Finally the essential differences between these 2 populations are that: a) the early one arises mainly from the YS and the late one mainly from the embryo; b) the intraembryonic organs are colonized essentially by cells of the late intraembryonic SC population. These facts, taken together, suggest that in the early phase the YS population becomes totally engaged in intense erythropoiesis, while the intraembryonic population remains dormant, ready for later needs.

In conclusion, YS erythropoiesis in the avian embryo is part of a complex scheme, involving several haemopoietic sites, several types of erythrocytes and a succession of overlapping Hbs. Morphological or biochemical study of normal development makes it possible to tie these factors together only partially. Our experimental approach, using quail chick 'YS chimaeras' yields a more precise picture of the contribution of the YS to the system. It demonstrates the importance of the intraembryonic blood islands, which participate in the sequential functioning of the mesodermal haemopoietic anlage. The first haemopoietic wave is extraembryonic, and occurs mainly in situ, without movements of SC. The 2nd wave is mainly intraembryonic; it involves very little in situ differentiation; the cells migrate to specific sites where they probably undergo commitment; one of these sites is the YS. The system is well adapted to ontogenic needs; it ensures rapid maximal production of erythrocytes at the early stage, and the diversification of cell lines at a later period.

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Microtubules, interkinetic nuclear migration and neurulation*

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Summary. The hypotheses dealing with mechanisms of neurulation are reviewed briefly. The phenomenon of interkinetic nuclear migration is thought to be an important factor to be considered in the invagination of the neuroepithelium in the chick embryo. Evidence is presented that implicates cytoplasmic microtubules in this phenomenon. It is suggested that microtubules not only participate in cell elongation but also that they are involved, through interkinetic nuclear migration, in the broadening of the basal region of the cells; this widening progressively creates the strain that ensures the invagination of the neuroepithelium.

Little is known of the many processes involved during the invagination of epithelial tissues. Yet, in many organs, normal morphogenesis includes the transformation of relatively flat epithelial leaflets into curved structures. This process of invagination is observed, for instance, during neurulation in many species[1–3], in the forming pancreas of the mouse[4] and in the early stages of development of the lens[5,6], salivary glands[7] and tubular glands of the chick oviduct[8].

Neurulation, as it occurs in the chick embryo, is well suited for an analysis of the events undergone by an epithelium in the course of its morphogenetic invagination. In the chick, the neural plate, a flat pseudostratified epithelial leaflet, invaginates first to take the form of a U-shaped structure (as seen in transverse sections) and then adopts a O-shape, which marks the closure of the neural tube (figure 1). Baker and Schroeder[1] report that in *Xenopus* changes in the shape of the cells making up the epithelium are basic to the tissue movement that ensures the closure of the neural tube. During neurulation the general aspect of the neuroepithelium is modified, while changes are observed in the shape of the cells making up the tissue. Indeed, in an early chick embryo, the cells found in the primitive streak region are cuboidal, while those forming the neural plate are of the low columnar variety (figure 1). Later in development, as the neural plate begins to invaginate to form the neural groove, cells will acquire a highly columnar form. In 1966, Waddington and Perry[9], studying neurulation in amphibians, postulated that cytoplasmic microtubules might have something to do with these changes in cell shape. In 1969, it was shown[10] that microtubules are oriented at random in the cuboidal cells that characterize the neuroepithelium of the primitive streak region of early chick embryos. Further, as development proceeds, more and more microtubules come to lie in a direction parallel to the long axis of the columnar cells that are characteristic of the later stages. The following year, Schroeder[11] noted that microtubules, which progressively orient themselves parallel to the cell's long axis, were instrumental in the production of the highly columnar shape of these cells in *Xenopus*. Using monoiodoacetamide coupled with cold exposure, it was possible to correlate experimentally the presence and orientation of microtubules with the columnar shape of the cells in the neural tube of the chick[12].

Baker and Schroeder[1] and Schroeder[11] proposed that, once the cells acquire their columnar shape, an apical ring of microfilaments may, in a purse-like fashion, constrict the cellular apices, thereby a) inducing a new shape referred to as bottle or flask shape, and b) assuring neurulation (figure 1). The proposal was tested