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# Development of the sickle canal, an unrecognized formation in the avian blastoderm, and its spatial relationship with the first appearing blood islands, induced by Rauber's sickle

**Marc Callebaut, Emmy Van Nueten, Fernand Harrisson and Hilde Bortier**

**University Antwerpen - RUC**
**Laboratory Human Anatomy & Embryology**
**Groenenborgerlaan 171, 2020 Antwerpen (Belgium)**

ABSTRACT. In the present study, we demonstrate the existence in avian blastoderms of a voluminous (approximately 2-4 mm long), previously unrecognized sickle-shaped canal (termed sickle canal). It usually bulges into the subgerminal space and is localized near the caudo-lateral border of the area pellucida after approximately one day incubation. The sickle canal, which is always visible on sections, is found both in the chicken and in the quail blastoderm. It seems to function as an expansion space for lateral migration of mesoblast cells, between epiblast and endoblast. The origin and evolution of the sickle canal have been followed (using quail-chick chimeras), by apposing quail Rauber's sickle fragments on fragments of unincubated chicken blastoderms. It was seen that part of the wall of the sickle canal is formed by endoblast derived from Rauber's sickle, i.e. transitional and junctional endoblast. Very obvious, on sections through the chimeras, is the intimate contact between the V or U-shaped quail junctional endoblast and the first formed blood islands, developing from mesoblast that migrates peripherally over the sickle canal. Our study demonstrates that even in the absence of the area opaca, a sickle canal forms and blood islands start to develop from mesoblast of the area pellucida under the influence of junctional endoblast (derived from Rauber's sickle). Rauber's sickle and its derivatives seem thus to be the major organizers of the avian blastoderm. During early incubation they induce the formation of endomesoblast ingressing via the primitive streak (CALLEBAUT & VAN NUETEN, 1994), and somewhat later junctional endoblast induces the development of blood islands from the most laterally ingressed mesoderm.

KEY WORDS : avian blastoderm, Rauber's sickle, sickle canal, junctional endoblast, blood islands.

## INTRODUCTION

Recent studies (CALLEBAUT, 1993a, b, c, 1994 ; CALLEBAUT & VAN NUETEN, 1994, 1995, 1996) yielded new data about the structures and developmental events in avian intra-uterine germs after bilateral symmetrization and in unincubated eggs. The terminology of the different components of an unincubated quail blastoderm and surrounding structures is represented in Fig. 1. The term Rauber's sickle (RAUBER, 1876) is used instead of Koller's sickle, since Rauber was the first to describe it (CALLEBAUT & VAN NUETEN, 1994). We used the term sickle endoblast because we have demonstrated that this part of the deep layer is directly derived from Rauber's sickle (CALLEBAUT & VAN NUETEN, 1994).

Corresponding author : M. Callebaut, e-mail : macal@ruca.ua.ac.be

The anti-sickle region (Fig. 1) was first described by CALLEBAUT (1993a) in gravitationally oriented quail germs. In this anti-sickle region, an irreversible disruption takes place between the future cranial part of the germ and the underlying subgerminal ooplasm at the moment of bilateral symmetrization (CALLEBAUT, 1993b, 1994). The anti-sickle itself is formed by a sickle-shaped group of loose yolk masses and cells located below the upper layer (UL) in the cranial recessus of the subgerminal space. The upper layer from the anti-sickle region of the unincubated chicken blastoderm is still uncommitted (CALLEBAUT & VAN NUETEN, 1995 ; CALLEBAUT et al., 1998a). Rauber's sickle divides the area pellucida into a peripheral caudal area marginalis and an area centralis. The area centralis contains a subgerminal space filled with liquid. By contrast, Rauber's sickle and the caudal marginal zone are directly in contact with

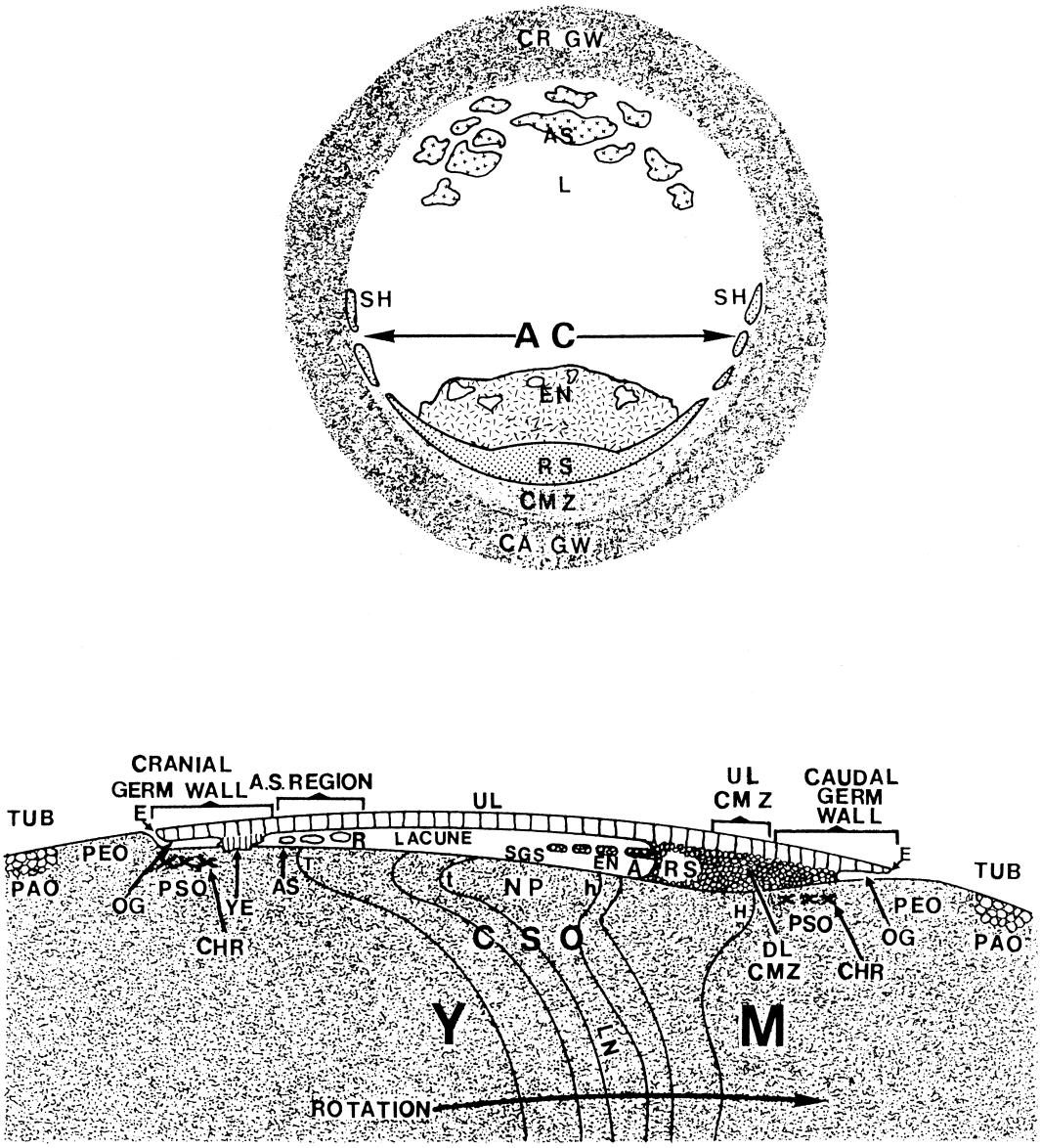



Fig. 1. – Top: Schematic representation of the components of the unincubated quail blastoderm seen from below after removal of the subgerminal ooplasm, ready for *in vitro* culture; CR GW: cranial germ wall; AS: anti-sickle region; L: lacune in the deep layer; EN: incomplete endophyll sheet; SH: fragmentary sickle horns; CMZ: caudal marginal zone more or less transparent; CA GW: caudal germ wall. AC: area centralis enclosed by Rauber's sickle (RS) and its sickle horns.

Bottom: Schematic representation of a mediosagittal section through an unincubated quail blastoderm with surrounding ooplasms after fixation *in situ* on the egg yolk ball. UL: upper layer; EN: incomplete endophyll layer; RS: Rauber's sickle; UL CMZ: upper layer from the caudal marginal zone; the caudal marginal zone being a more or less transparent part adherent to the caudal peripheral subgerminal ooplasm (PSO) via a deeper part (DL CMZ); SGS: subgerminal space forming a caudal pocket A (axilla shaped) and a cranial recess (R) in which free yolk masses or sometimes cells are found forming the anti-sickle (AS); E: edge of the blastoderm; OG: early overgrowth zone (Callebaut & Meeussen, 1988); YE: early development of the yolk endoblast, growing into the peripheral subgerminal ooplasm (PSO); CHR: chromosome clusters; PEO: perigerminal ooplasm; PAO: paragerminal ooplasm forming a tubulin-rich ring (TUB) at distance from the edge of the blastoderm (Callebaut et al., 1996b); YM: the voluminous yolk mass of the egg yolk ball in which the eccentricity of the successive yolk layers parallel with the eccentricity in the blastoderm is represented; CSO: central subgerminal ooplasm in which the central nucleus of Pander (NP) (Pander, 1817) is seen; t: toe-shaped and h: heel-shaped part of the nucleus of Pander; T: toe-shaped and H: heel-shaped part of the surrounding yolk layers as result of the rotation *in utero* (the arrow indicates the direction of rotation and compression of the yolk mass under the combined influence of gravity and egg rotation) (Callebaut, 1983, 1993a); LN: bent latebra neck. Note that by contrast to the caudal germ wall, the cranial germ wall is disrupted from the underlying peripheral subgerminal ooplasm (Callebaut, 1993, a, b, c).

the caudal underlying peripheral subgerminal ooplasm without underlying cavity. In the caudo-central region of the area centralis a more or less developed sheet of endophyll can be seen. We used the name endophyll (CELESTINO DA COSTA, 1948), and not primary hypoblast, to distinguish the endophyll from Rauber's sickle *Anlage*, which appears earlier or at the same time (CALLEBAUT, 1993a, 1993c, 1994). Previous studies (CALLEBAUT & VAN NUETEN, 1994; CALLEBAUT et al., 1996a) indicate that the main function (i.e. definitive endoderm and mesoderm induction in the upper layer) of Rauber's sickle in avian blastoderms is homologous to the function of Nieuwkoop's centre (NIEUWKOOP, 1969, 1973) in amphibian blastulas. CALLEBAUT & VAN NUETEN (1995) have shown that endophyll orients the direction of the primitive streak, starting from Rauber's sickle. Although Rauber's sickle-derived material (sickle endoblast and junctional endoblast) and endophyll have a very important and indispensible inductive function for the development of the definitive embryonic tissues (which are all derived from the upper layer) during gastrulation and neurulation, they never give rise to any definitive structure and therefore belong to the so-called extraembryonic part of the blastoderm. The junctional endoblast forms a whitish structure and can be easily seen at the surface of the living blastoderm *in situ* on the egg yolk ball of a quail egg after 11-13h incubation (Fig. 2). It forms the V or U-shaped part of the deep layer (visible through the transparent UL) in the early avian primitive streak embryo. It is derived from Rauber's sickle cells that migrate in the neighbouring ooplasm during early incubation (CALLEBAUT & VAN NUETEN, 1994). The angle formed by the junctional endoblast is bissected by the primitive streak formed in the upper layer (Figs 2, 3). Laterally from the convexity of the junctional endoblast one can distinguish the yolk endoblast, which has a less dense white aspect but which is more voluminous than the junctional endoblast. The yolk endoblast is localized in the area opaca. The junctional endoblast that forms *in situ* from the Rauber's sickle (CALLEBAUT & VAN NUETEN, 1994), has strong embryo-inducing and dominating potencies (CALLEBAUT et al., 2000a). In the present study, we observed that during avian neurogastrulation, an unidentified sickle-shaped canal (called "sickle canal") develops parallel with the V-shaped junctional endoblast by caudal fusion. Since the general shape and localization of the sickle canal strongly resemble the localization of the first appearing group of blood islands in the avian blastoderm, as described by SETTLE (1954), we tried to find out if there is a relationship between the sickle canal and early

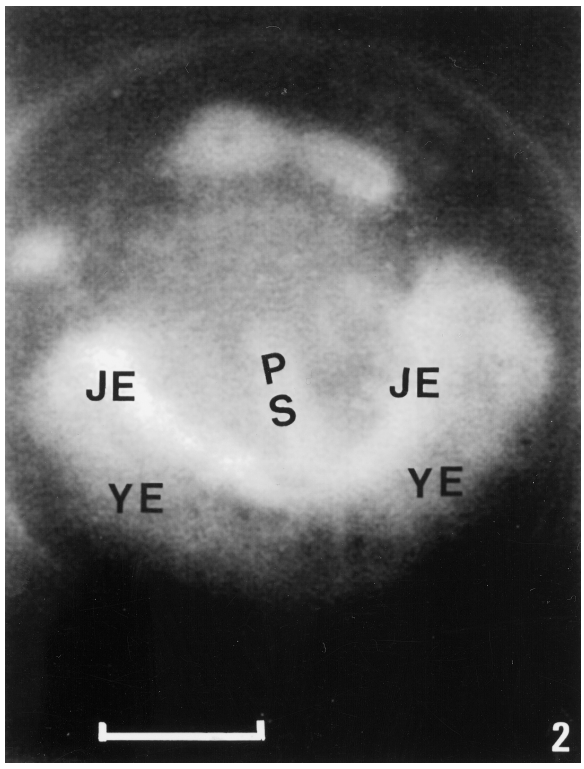



Fig. 2. – Stereomicroscopic photomicrograph of a living quail embryo (Stage HAMBURGER & HAMILTON, 1951) *in situ* on its egg yolk ball, incubated for 12h; PS primitive streak; JE: junctional endoblast; YE: yolk endoblast; bar: 1mm.

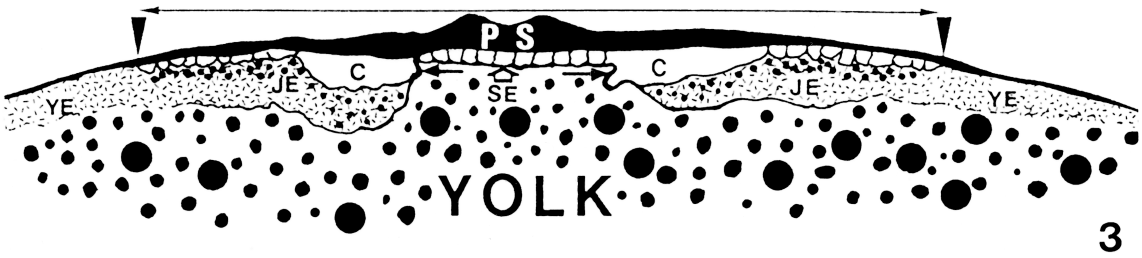



Fig. 3. – Schematic drawing of a transverse section through the primitive streak region (localized between the two vertical arrowheads as a region where the upper layer (UL) is thicker than laterally) of a stage 3+ quail embryo (HAMBURGER & HAMILTON, 1951); between the deep side of the primitive streak (PS) and the sickle endoblast (SE) indicated by a vertical hollow arrow, there are cellular extensions passing between small cavities. The horizontal arrows on the left and the right indicate the transitional endoblast (CALLEBAUT & VAN NUETEN, 1994) which connects the sickle endoblast with the junctional endoblast (JE); C: pararchenteric canals; between the lateral part of the junctional endoblast and the deep side of the primitive streak region there are numerous extensions and small cavities, whilst between the more lateral yolk endoblast (YE) and the UL, there are no cavities and no extensions.

erythropoiesis. An inductory influence of endoderm (endoblast) on the development of blood islands was suspected already at the head-process stage (revised by HAMILTON, 1965). WILT (1965) and MIURA & WILT (1969) have shown that stage 4 (HAMBURGER & HAMILTON, 1951) area opaca vasculosa (AOV) endoderm has a salutary influence on the erythropoietic differentiation of stage 4 AOV ectoderm-plus-mesoderm explants cultured on egg-white-agar medium or on plasma clots on the same or opposite sides of millipore filters. ZAGRIS (1982) has shown that multiple vascular areas (usually with a U-shaped configuration, resembling the area opaca vasculosa) are formed in unincubated or prestreak chicken blastoderms under the influence of multiple transplanted hypoblasts. PARDANAUD et al. (1996, 1999) have shown by means of quail/chick transplantation that two subsets of the mesoderm give rise to endothelial precursors: a dorsal one, the somite, produces pure angioblasts (angiopoietic potential), while a ventral one, the splanchopleural mesoderm, gives rise to progenitors with a dual endothelial and hemopoietic potential (hemangiopoietic potential). In most studies of early avian erythropoiesis, the authors describe a stimulatory influence of endoblast on the formation of blood islands. However, the source of this endoblast (originally derived from the area opaca or not?) is not mentioned or is not known. In the present study, using quail-chick chimeras, we demonstrated that the bottom of the sickle canal is formed by junctional endoblast. The first blood islands develop in the most lateral part of the mesoblast when it slides over this junctional endoblast, borderzone between area pellucida and area opaca, just peripherally from the sickle canal. This suggests an induction effect of the junctional endoblast on the mesoblast to form the first blood islands.

## MATERIAL AND METHODS

### Stereomicroscopic and histological observations on sections of primitive streak blastoderms of quail or chicken

Eggs from chicken or quail (*Coturnix coturnix japonica*) were incubated at 38-39°C during 13-30h.

A) Some of these blastoderms were removed from their egg yolk balls, observed and photographed in the living state. Thereafter, they were fixed in calcium-formalin or in Susa without sublimate (ROMEIS, 1948) for 1 night.

B) Other blastoderms remained *in situ* on their egg yolk balls. Some of their structures, visible from the surface, were labelled by placing charcoal particles or carmine on the surface of the vitelline membrane. The blastoderms still *in situ* on their egg yolk balls were fixed in the same fixatives as in A.

C) From other unincubated chicken or quail eggs the blastoderms were removed and cultured *in vitro* for 20-60h according to the technique of NEW (1955). Instead of Petri dishes, the culture vessels described by GAILLARD (1949) on which an optically flat glass cover was sealed with hot paraffin, were used. Fixation was performed in Susa without sublimate (ROMEIS, 1948) for 1 night (as mentioned above). After fixation, all the isolated blastoderms or blastoderms still *in situ* on their egg yolk balls were placed in tap water and progressively dehydrated in an alcohol series. The blastoderms, which were still on their egg yolk ball, were excised together with some subgerminal ooplasm in the absolute alcohol bath. After clearing in xylene, all the blastoderms were embedded in paraffin. The blastoderms were sectioned perpendicularly to their caudocephalic axis. After deparaffination, the sections were stained with Unna (after SILVERTON & ANDERSON, 1961) or with iron hematoxylin and eosin.

### Study of chimeras developing under influence of quail Rauber's sickles placed on unincubated chicken blastoderm parts in culture

This was done to determine the origin and to follow the development of the sickle canal and associated structures (i.e. the embryonic blood islands). The experimental procedure used (placing quail Rauber's sickle fragments on parts of unincubated chicken blastoderms) is represented in a scheme accompanying each of the photomicrographs of the studied chimeras. The quail-chicken chimeras were cultured for 24-31h according to NEW (1955) or according to SPRATT (1947). In the latter case the culture medium was not pure egg white, but a mixture of 25ml thin egg white and a gel made of 150mg Bactoagar (Difco, Detroit, Mi) in 25ml Ringer's solution. This semi-solid medium allowed microsurgery and further culturing on the same substratum. Stereomicroscopic polaroid photographs were taken in the same direction at the beginning, during and at the end of the culture period. After fixation, dehydration and embedding in paraffin wax as described above, the chimeric blastoderms were sectioned perpendicularly to the visible or presumed axis. The deparaffinized, 8-µm-thick sections were Feulgen-stained after DEMALSY & CALLEBAUT (1967) in order to identify the origin of the nuclei, using microscopic objectives x10 or x20. This allowed us to observe the typical central or subcentral chromatin granule of the grafted quail cells (CALLEBAUT, 1968; KOSHIDA & KOSIN, 1968; LE DOUARIN & BARQ, 1969), as well as to gain an overview of the distribution of the quail cells among the chicken cells. Some sections were stained with iron hematoxylin and eosin. The photographs of sections of cultured blastoderms or chimeras were represented with the deep layer directed downwards although they were cultured with the deep layer upwards.

## RESULTS

### Stereomicroscopic and histological observations

*After removal of the blastoderm from its egg yolk ball*

Under the stereomicroscope with incident light illumination, at the lower surface of a living primitive streak blastoderm, sometimes a V-shaped transparent canal can

be seen (Fig. 4). Both sides (each approximately 1mm long) of the canal converge on the caudal midline forming a valve-like structure. The whole sickle canal, seen from the deep side, takes the aspect of a uterus bicornis. Very close to and immediately behind and lateral of the membranous part of the canal, a dense V-shaped structure can be seen. It is formed of junctional endoblast (confirmed on sections). Both sides of the sickle canal end blindly cranially, and no connections are seen with the cranial endophyllic (germinal) crescent. The cranial ends of the sickle canal do not reach the level of Hensen's node. On a low power view of a section through such a blastoderm after fixation and staining (Fig. 5), the gross morphology of the sickle canal and neighbouring structures can be observed. In a transverse section, the lumen of the sickle canal has a diameter of approximately 200µm. Laterally, the lumen extends as a narrow slit above the junctional endoblast. The bottom of the sickle canal is formed medially by the thin transitional endoblast (CALLEBAUT & VAN NUETEN, 1994) and laterally by the massive junctional endoblast. The roof of the canal is formed by mesoblast extending laterally above the junctional endoblast as a thickening (forming blood islands below the epiblast). The border zone between area opaca and area pellucida is formed by junctional endoblast (derived from Rauber's sickle material). In about half of the isolated blastoderms, no sickle canals are seen from their surface *in toto*. However after sectioning of these blastoderms, it was seen that the sickle canals were always present, sometimes with a less voluminous or asymmetric lumen, or disrupted bottom. In somewhat older blastoderms (Fig. 6), the canals still remain visible but become proportionally smaller.

*After fixation in situ on the egg yolk ball*

Sections through the caudal region of intermediate streak blastoderms (approximately 13h incubation, corresponding to stage 3 of HAMBURGER & HAMILTON, (1951)) invariably show pararchenteric canals, that form bilaterally an intraembryonic space between mesoblast and deep layer (Fig. 7). The bottom of these canals is first flat, but later on it usually bulges in the subgerminal cavity. The latter contains a liquid, which after fixation forms a cast adhering tightly to the thin sickle endoblast (medially) and to the junctional endoblast (laterally). On sections through the caudal part of older blastoderms (stage 7 of HAMBURGER & HAMILTON, 1951), the sickle canal composed of two asymetric pararchenteric canals can always be seen (Fig. 8). In this most caudal part a narrow connection exists (separated by an incomplete septum) between right and left (Figs 8, 9). In this caudal region, the sickle endoblast is not in direct contact with the ingressing mesoderm, as is the case in more cranial regions. The median sickle endoblast is in intimate contact with and seems tightly adherent to the underlying coagulated contents of the subgerminal cavity (Fig. 9). This tight contact is probably the reason the sickle canal is sometimes no longer visible after dissection from below in the living blastoderm. Laterally, the junctional endoblast is seen (Fig. 10) above which blood islands are forming in the mesoblast localized in the immediate neighbourhood. Also blood islands are seen above the more laterally-localized yolk endoblast in the area opaca. Immediately medial to the junctional endoblast region (at the level of the sickle canal), the blastoderm is formed only by three thin layers (Fig. 10): epiblast, mesoblast and sickle endoblast or transitional endoblast. So, the localization of the sickle canal can usually be seen and labelled from the surface of the living blastoderm as a V-shaped transparent zone. By apposing charcoal particles on it *in vivo*, the latter can be recognized on sections (Fig. 10).

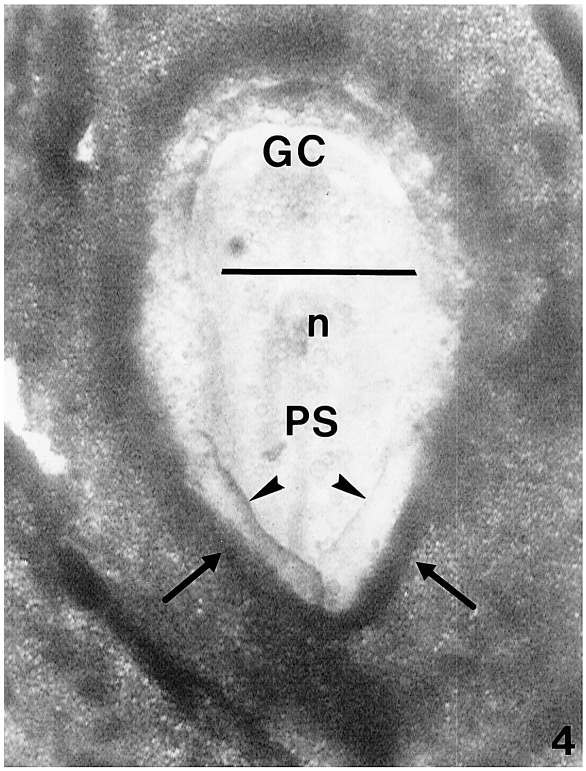
*Observations on the development of the sickle canal in cultured blastoderms (starting from the unincubated stage)*

After culture for 24-29h, the sickle canal is usually clearly visible (if not hidden by subgerminal contents) behind the caudal part of the embryo (Fig. 11). Frequently lateral extensions of the lumen of the sickle canal are seen. In sections, the latter are seen to extend into the area opaca. At certain stages of development the sickle canal of cultured blastoderms is visible as a broad, flat sac (Figs. 11, 12). After prolonged culture (more than two days), the sickle canal is usually still observed (Fig. 13).

**Study of chimeras developing under influence of quail Rauber's sickles placed on parts of unincubated chicken blastoderms in culture**

A) In a first experimental group (n=8) the caudo-lateral rim zone including the caudal marginal zone, Rauber's sickle and part of the neighbouring area centralis were removed from an unincubated chicken blastoderm (Fig. 14A). This avoids possible interference with the autochthonous chicken Rauber's sickle. Subsequently a quail Rauber's sickle fragment was placed on the anti-sickle region close to the cranial marginal zone. The stereomicrograph (Fig. 14B) shows a chimera, as represented schematically in Fig. 14A, at the onset of the culture. After 29h, a normal embryo has developed with caudocephalic axis starting from the place where the quail Rauber's sickle fragment was placed, i.e. in a diametrically opposed direction to the presumed original polarity of the chicken blastoderm (Fig. 14C). On sections through the caudal part of this chimeric embryo (Fig. 14D) the formation of a chicken blood island is seen as a swelling of the most lateral extension of the peripherally-migrating mesoblast, in the immediate neighbourhood of the quail junctional endoblast. The latter surrounds the chicken blood island from below. From medially to laterally, the chicken blood island has a denser aspect with numerous mitotic figures.

B) In a second experimental group (n=9), the central part of the area centralis of unincubated chicken blastoderms was sectioned circularly (caudally, at some dis-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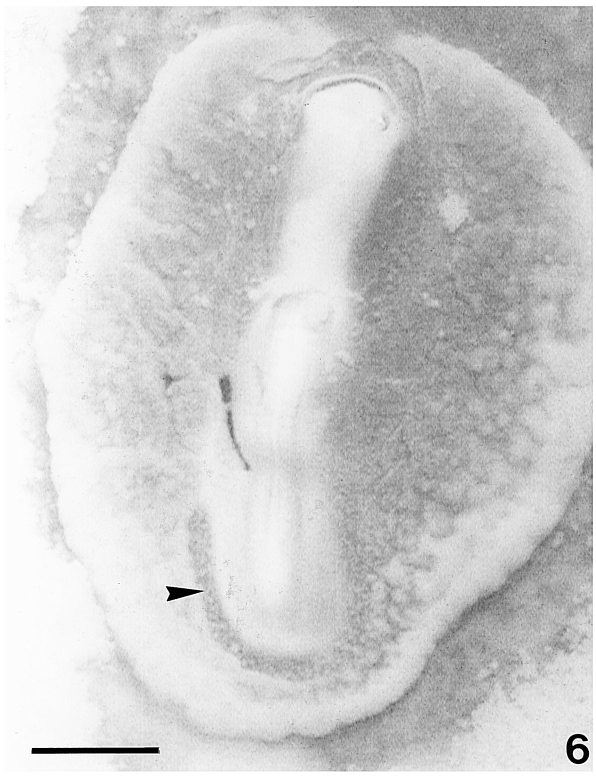
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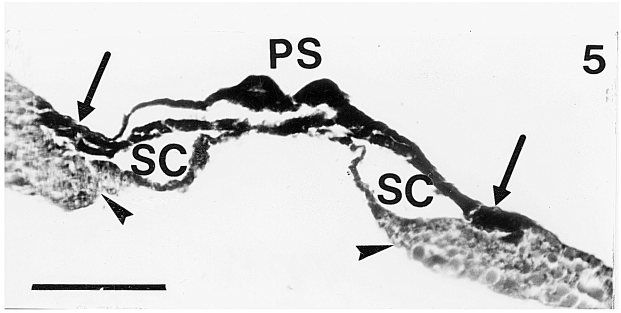
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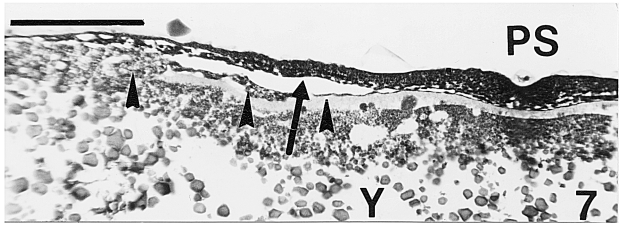
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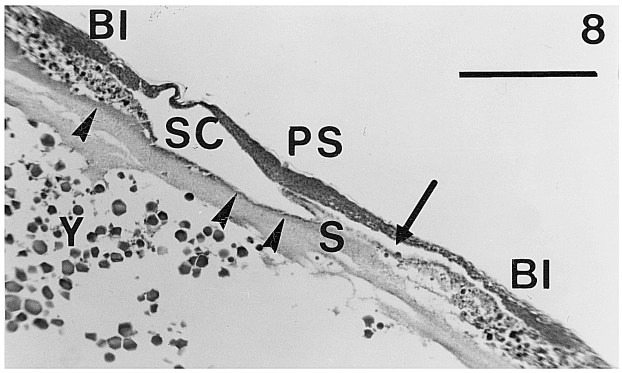
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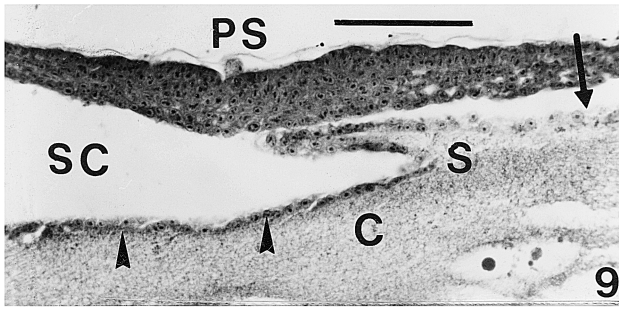
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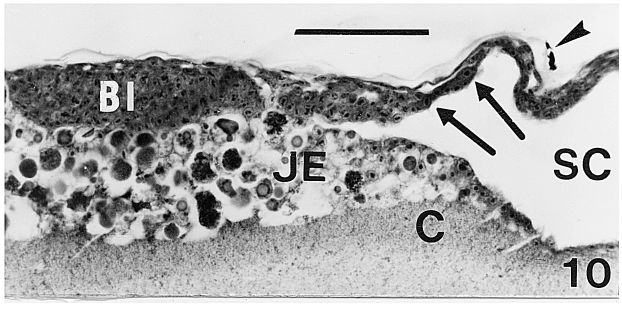
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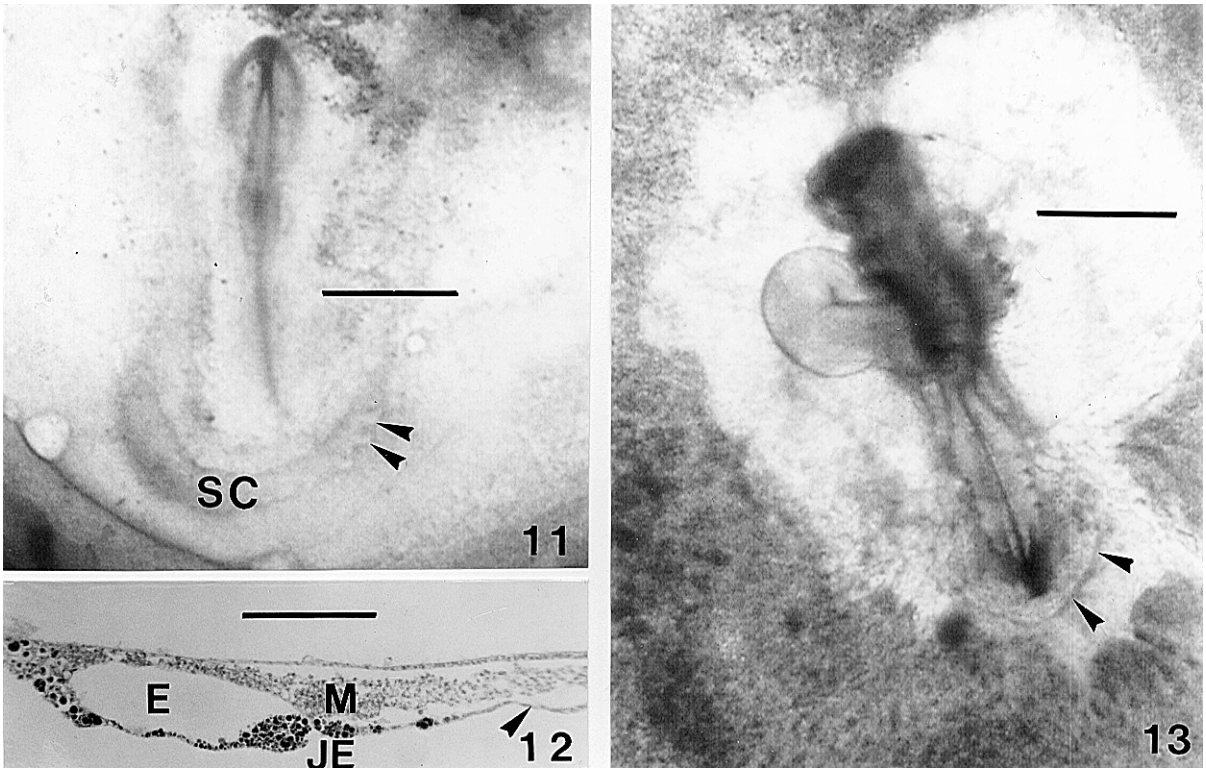



Fig. 4. – Stereomicrograph of a living quail primitive streak blastoderm incubated for 20h (corresponding with a chicken stage 5 of HAMBURGER & HAMILTON, 1951) and removed from its egg yolk ball; as seen from its deep side: the V-shaped thin-walled transparent sickle canal (arrowheads) is very obvious; arrows indicate junctional endoblast on each side; n: Hensen's node; PS: primitive streak; GC: germinal crescent formed by cranially displaced endophyll; oblique illumination; bar: 1mm.

Fig. 5. – Transversal section through a quail blastoderm of the same age as represented in Fig. 4, after fixation in calcium-formalin and staining with Unna; note the presence of the sickle canal (SC) on both sides; PS: primitive streak region; the arrowheads indicate junctional endoblast; the arrows indicate early development of blood islands from mesoblast that migrates peripherally over the junctional endoblast and below the epiblast. Note that one sickle canal would be hidden by the junctional endoblast when observed from below; bar: 200µm.

Fig. 6. – Deep side of fixed quail blastoderm after removal from its egg yolk ball after 31h incubation (corresponding with a chicken stage 7 of HAMBURGER & HAMILTON, 1951); sickle canal indicated by arrowhead; oblique illumination; bar: 1mm.

Fig. 7. – Section through the caudal region of a quail intermediate streak blastoderm (corresponding to stage 3 of HAMBURGER & HAMILTON (1951) in the chicken) fixed *in situ* on its egg yolk ball; PS: primitive streak region; the arrow indicates the lumen of the pararchenteric canal; the arrowheads indicate, from medially to laterally: sickle endoblast, transitional endoblast and junctional endoblast; Y: yolk mass; iron hematoxylin and eosin staining; bar: 200µm.

Fig. 8. – Section through the caudal part of a quail embryo after 23h incubation, (corresponding to a chicken stage 6 of HAMBURGER & HAMILTON, 1951) fixed *in situ* on its egg yolk ball (Y); PS: caudal part of the primitive streak (plate); SC: the lumen of one side of the sickle canal is very wide, whilst the lumen of the other side (indicated by arrow) is narrow; both lumina are separated by an oblique incomplete septum (S); BI: onset of formation of blood islands below the epiblast; the subgerminal space contains a coagulate, which is tigthly fixed to the sickle endoblast or junctional endoblast (arrowheads): iron hematoxylin and eosin staining; bar: 300µm.

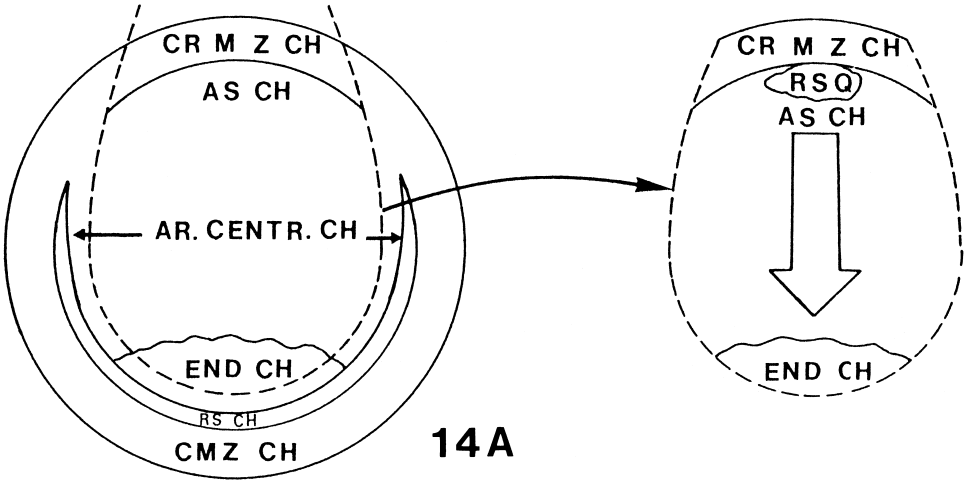
Fig. 9. – Higher magnification of the medial part of Fig. 8; same indications as for Fig. 8; C: coagulate in the subgerminal space, tightly adhering to the sickle endoblast; iron hematoxylin and eosin staining; bar: 100µm.

Fig. 10. – Enlarged view of the lateral part of Fig. 8; SC: lumen of sickle canal: BI: blood island; the arrows indicate mesoblast migrating over the junctional endoblast (JE), forming a blood island; the arrowhead indicates a charcoal particle that was placed *in vivo* on the transparent V-shaped zone visible from the surface; bar: 100µm.

Fig. 11. – Stereomicrograph of a living chicken blastoderm (stage 8 of HAMBURGER & HAMILTON, 1951) after 28h of culture. The sickle canal (SC) has a length of approximately 4 mm; some lateral extensions (indicated by arrowheads) are visible; bar: 2 mm.

Fig. 12. – Section through a lateral extension cavity (E) of the sickle canal seen in Fig. 11. Note the thickening of the mesoblast layer (M) above the junctional endoblast (JE); sickle endoblast is indicated by an arrowhead; Unna staining after calcium-formalin fixation; bar: 200µm.

Fig. 13. – Stereomicrograph of a living chicken embryo after 48h of culture (stage 14 of HAMBURGER and HAMILTON, 1951); the sickle canal (indicated by arrowheads) is still visible; bar: 1mm.

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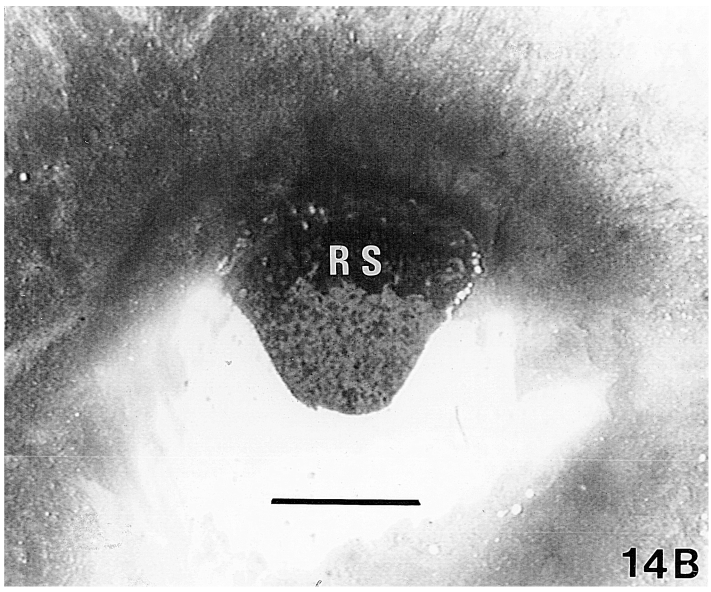
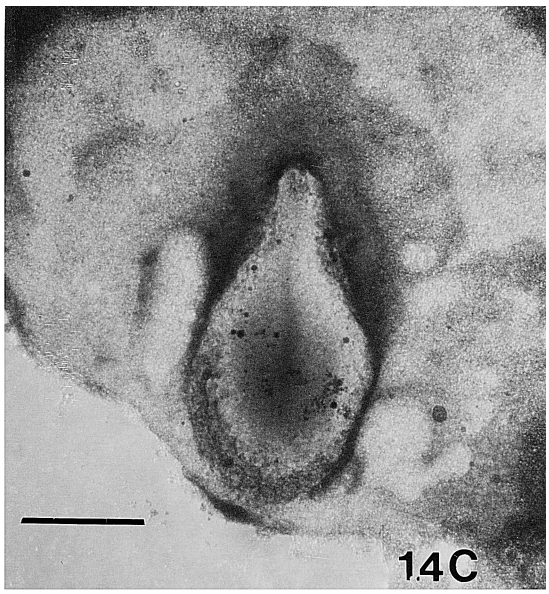
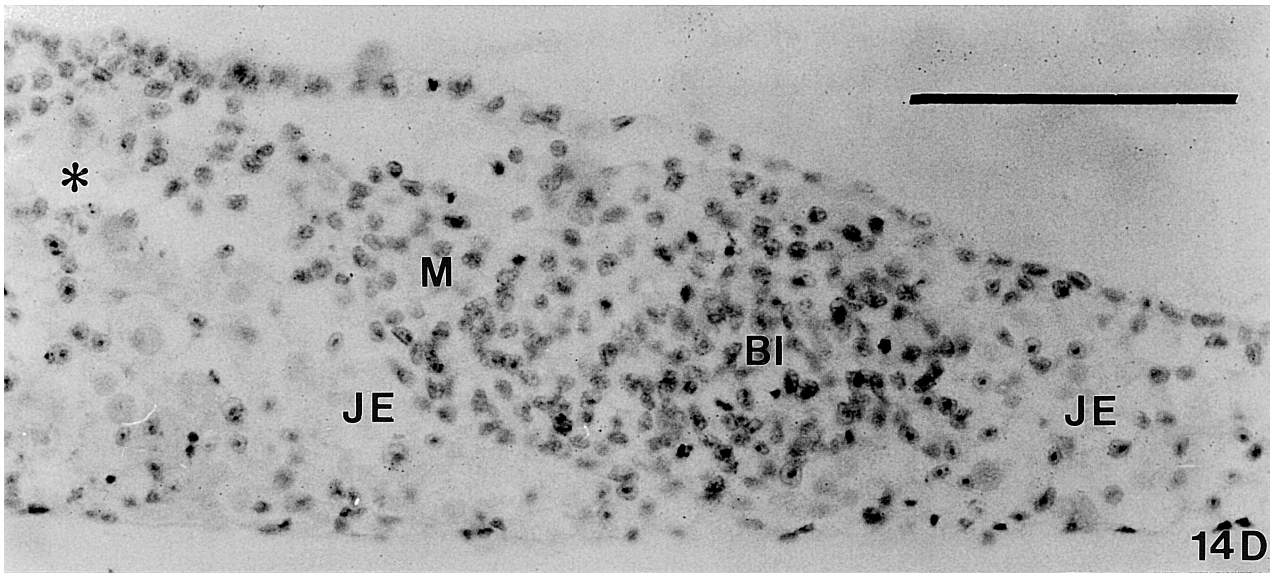



14B



14C




14D

tance from and parallel to Rauber's sickle) (Fig. 15A). On the cranial zone of this central part of the chicken area centralis, which caudally contains some endophyll, a fragment of a quail Rauber's sickle was placed. The photomicrograph (Fig. 15B) shows a chimera, as represented schematically in Fig. 15A, at the start of the culture. After 25h of culture, an embryo has developed (Fig. 15C), again with a caudo-cephalic axis starting from the place where the quail Rauber's sickle was placed, i.e. in a diametrically opposed direction to that originally programmed, had the autochthonous Rauber's sickle been left in place. Fig. 15D, shows a section through the caudal region of the embryo of Fig. 15C. A blood island is forming above the sickle canal. It originates from the most lateral part of the peripherally migrating chicken mesoblast where it comes in contact with the quail junctional endoblast. All the blood islands (formed from chicken cells) seen in these embryos are in close relationship with the quail junctional endoblast. This demonstrates that even in the total absence of area opaca but in the presence of a Rauber's sickle, a sickle canal and blood islands can develop in the area pellucida.

**Legends to the figures** (see opposite page)

Fig. 14A. – On the left: Schematic drawing representing the incision (indicated by a dotted line) in an unincubated chicken blastoderm, with the aim to remove its caudolateral rim zone including the caudal marginal zone, Rauber's sickle and part of the neighbouring area centralis; CR MZ CH: cranial marginal zone from chicken; AS CH: anti-sickle chicken; AR. CENTR CH: area centralis chicken; END CH: remaining endophyll chicken; RS CH: Rauber's sickle chicken; CMZ CH: caudal marginal zone chicken.
On the right: Scheme representing a quail Rauber's sickle fragment (RSQ) placed on the anti-sickle region (AS CH) of the remaining part of the unincubated chicken blastoderm as represented on the left; the large empty arrow indicates the caudo-cephalic axis of the future embryo that will develop from the apposed quail Rauber's sickle in the direction of the endophyll of the chicken.

Fig. 14B. – Stereomicrograph of a chimera, as obtained according to the procedure represented on Fig. 14A, at the onset of the culture; RS: apposed Rauber's sickle of quail; bar: 2mm.

Fig. 14C. – Stereomicrograph of the chimera of Fig. 14B after 29h of culture: a normal embryo has developed with diametrically opposed direction of the caudo-cephalic axis as could be predicted (see Fig. 14A); bar: 1mm.

Fig. 14D. – Section through the caudal region of the chimera of Fig. 14C; BI: chicken blood island developing from the most lateral part of the chicken mesoblast (M) under influence of the neighbouring quail junctional endoblast (JE); numerous mitotic figures are seen in the blood island; *: lateral extension of the sickle canal; Feulgen staining; bar: 100µm.

## DISCUSSION

Neither HAMBURGER & HAMILTON (1951) nor HAMILTON (1965) describe the sickle canal.

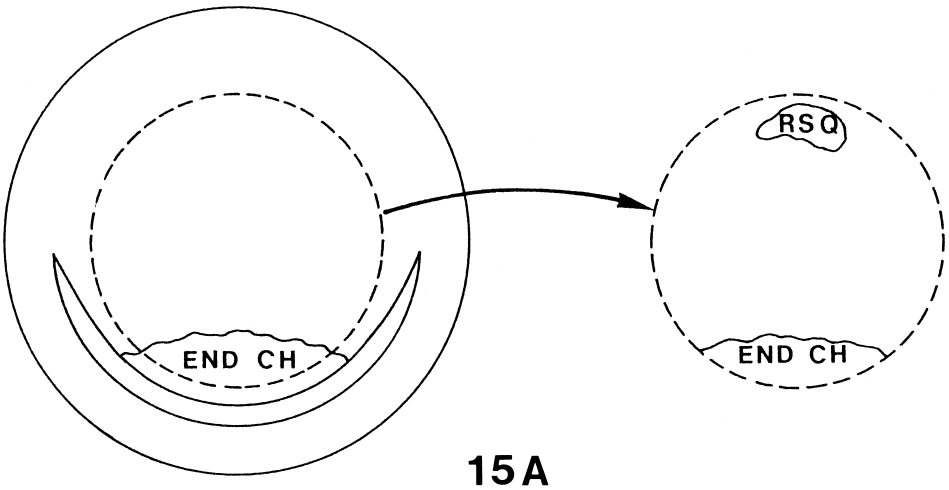
Also in more recent general descriptions of avian embryology (BELLAIRS, 1991, BELLAIRS & OSMOND, 1998), the sickle canal is not mentioned. In the present study, we show that the sickle canal is not a fixation artifact, since it can be observed in the living state. Moreover after fixation of the blastoderm, still *in situ* on its egg yolk ball, the sickle canal or the pararchenteric canals are always seen on sectioned material. After *in vivo* surface labelling with charcoal on a transparent V-shaped zone of a blastoderm of approximately one day incubation, its corresponding localization as sickle canal can be identified on the sections. The sickle canal has until now not been recognized as such for several reasons:

1) Since the endoblastic wall of the sickle canal seems to be tightly fixed to the coagulate present in the subgerminal cavity, its observation can be impaired by adhering coagulate. For the same reason, the very thin deep wall of the sickle canal can be disrupted during dissection from the lower side.

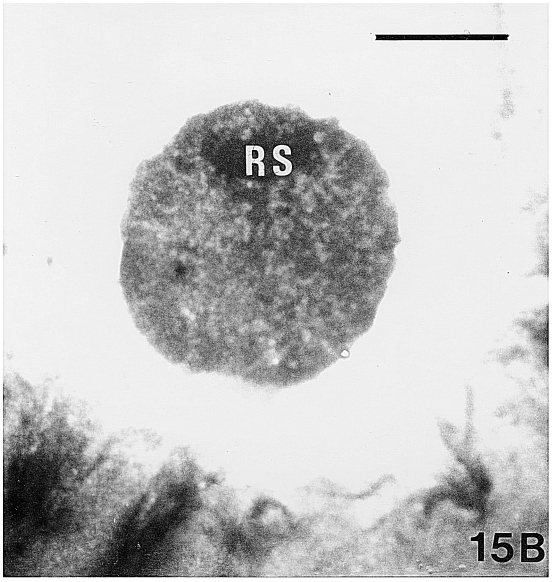
2) The sickle canal is sometimes wholly or partially obscured from its deep side by the neighbouring, also sickle-shaped junctional endoblast (see Fig. 5).

3) The sickle canal is often not clearly visible in transmitted light. By the use of oblique incident light we obtained a much better visualization of this structure.

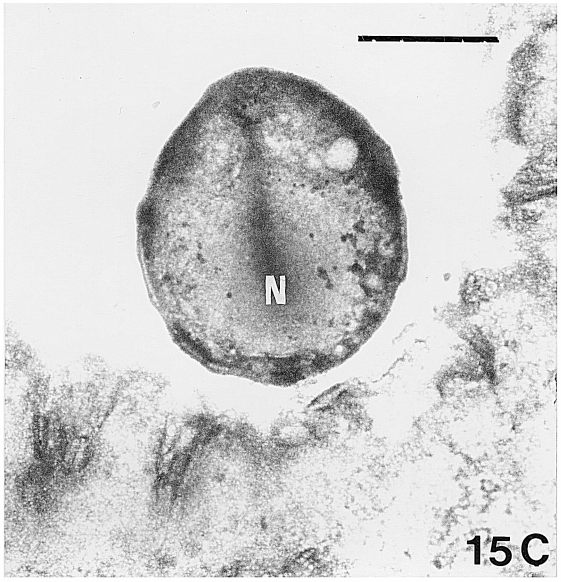
That the sickle canal is not always visible from the surface of the whole blastoderm but always observed on sectioned material can be compared with the visibility of Rauber's sickle, with which it is directly related. Indeed Rauber's sickle, already described by RAUBER in 1876, is also not always easily seen from the exterior of the egg yolk ball or after removal of the blastoderm (CALLEBAUT et al., 1998b). Therefore, for decades its existence was a matter of dispute or totally ignored. It was only in 1994 that CALLEBAUT & VAN NUETEN demonstrated its fundamental importance for the organization of the avian blastoderm during gastrulation. Moreover in the present study we demonstrated that also blood islands are formed under its inductory influence. The extraembryonic mesoderm of the chick embryo originates from the primitive streak and spreads out invasively between ectoderm and endoblast (GRODZINSKI, 1934; FLAMME, 1989). From the second day of incubation (in the chicken) onwards, the area occupied by mesoderm is called area vasculosa due to its abundant vascularization. The surrounding area that is still free of mesoderm is called area vitellina. The area vasculosa (after 2-3 days incubation) is subdivided into two concentric zones, which are named according to their optical properties: the inner transparent area pellucida vasculosa in the center of which the embryo lies, and the surrounding less transparent area opaca vasculosa, which is peripherally limited by the sinus terminalis. The different optical prop-



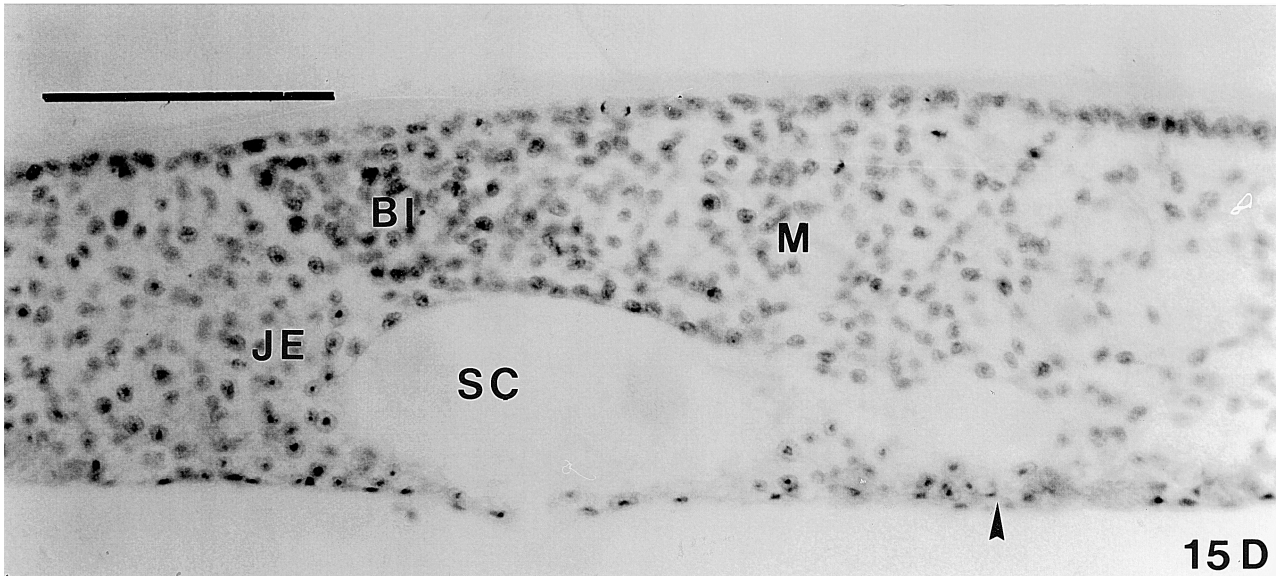

15A




15B




15C




15D

erties of these zones are due to the differing morphology of the endoblast cells: the cells of the area pellucida vasculosa endoblast are flat, while those of the area opaca vasculosa endoblast are high-prismatic and filled with large yolk vacuoles. According to FLAMME (1989) the endoblast cells of the area pellucida vasculosa and area opaca vasculosa are two different populations. The microvilli-bearing flat cells at the periphery of the area pellucida vasculosa are possibly descendants of the primary hypoblast (WAKELY & ENGLAND, 1978), lining the definitive endoderm, which invades from the upper layer through the primitive streak and which during expansion withdraws the primary hypoblast to the periphery. The origin of the erythropoietic stem cells has been analysed in avian chimeras by DIETERLEN-LIÈVRE et al (1976), DIETERLEN-LIÈVRE (1978), BEAUPAIN et al (1980) and PARDANAUD et al. (1987, 1989). These studies suggested that the first red blood cells develop in the area opaca. Therefore, the term "yolk sac" erythropoiesis was used. It encompasses red cell production by this individualized wall in which indeed big numbers of blood islands and erythrocytes appear. This suggested that the primary source of erythrocytes was localized in the area opaca, containing yolk endoblast. However, the experiments of these authors were performed by grafting the central part of the area pellucida of a quail blastoderm into the area opaca of a chick blastoderm around 30h of incubation, according to the technique of MARTIN (1972), or in still older stages. This is later than the moment when the first blood islands become visible (18-22h of incubation) at the head fold stage (HAMILTON, 1965). So, in their experiments, already a migration of the most peripheral mesoblast of the chicken area pellucida into the chicken area opaca had taken place, which could explain why they found only chicken erythrocytes in the circulation of the chimeras during the first days of embryonic life. Our experiments with quail-chick chimeras were, however, performed much earlier, starting with parts of unincubated blastoderms. After placing a quail Rauber's sickle fragment on the central region of the area centralis of an unincubated chicken blastoderm and culture, we observed the formation of blood islands in the total absence of area opaca material. All the chick blood islands, in the formed chimeric embryos, developed in close association with quail junctional endoblast. SETTLE (1954), in a series of experiments with circular pre-streak blastoderm fragments (incubated for several hours and not unincubated as in our experiments) placed in culture, clearly revealed that erythrocytes are never formed from culture of the area opaca only. When a circular cut is made inside the boundary between the area opaca and the area pellucida (where in our study Rauber's sickle or junctional endoblast is present), he was able to demonstrate the appearance of hemoglobin in both the inner and outer pieces. However, as the ring of incision approaches the center of the blastoderm, hemoglobin appears less frequently in the central piece. After culture, no hemoglobin is formed in the isolated cranial third of a prestreak embryo with visible embryonic shield (SETTLE, 1954). So, the results of SETTLE (1954) can be explained by at least a starting inductory influence of junctional endoblast on the formation of blood islands or by the absence of junctional endoblast in the cranial and/or central regions of the blastoderm. In a study of the differentiation of the yolk sac endoderm of the chicken embryo, in which cysteine lyase was observed to be a marker of differentiation, BENNETT et al. (1972) and BENNETT (1973) suggested that the mesoderm exerts an influence on the yolk sac endoderm, resulting in the differentiation of this tissue and the appearance of cysteine lyase. However, a relationship between the appearance of cysteine lyase in the yolk sac endoderm of the chicken embryo, and the presence of the blood-forming, mesodermal component, i.e. the area vasculosa, in the extraembryonic area could not be confirmed (VAN ROELEN & VAKAET, 1983). Also the assumption of a primary mesodermal subdivision into area opaca vasculosa and area pellucida vasculosa is inconsistent with the observation that a zonal subdivision exists in the endoblast long before it is overgrown by the mesoderm migrating outward from the primitive streak (PS) (BELLAIRS, 1963; ENGLAND & WAKELY, 1977). The earlier emergence of blood islands at the border of the area pellucida, earlier than shown in previous studies does not challenge the observation that the first erythrocytes emerge in the yolk sac: indeed, the migration of the mesoderm, laterally, also involves the migration of blood islands, laterally in the yolk sac before the onset of the circulation. Our

**Legends to the figures** (see opposite page)

Fig. 15A. – On the left: Schematic representation of the isolation of the central part of the area centralis of an unincubated chicken blastoderm, by a circular incision (dotted line) at some distance from the internal border of Rauber's sickle. Thereafter, the whole area marginalis, Rauber's sickle and the peripheral part of the area centralis were discarded.
On the right: On this isolated central part of the area centralis (which is composed of upper layer with some chicken endophyll (END CH), (only localized in the original caudal part of the blastoderm), a quail Rauber's sickle fragment (RSQ) is placed, near the anti-sickle region.

Fig. 15B. – A chimera as represented schematically in Fig. 15A, at the beginning of the culture; RS: Rauber's sickle fragment from quail; bar: 1mm.

Fig. 15C. – The same chimera as in Fig. 15B after 25h of culture (before fixation); an embryo has developed with a straight primitive streak starting from the region where the quail Rauber's sickle fragment was placed: a neural plate (N) is seen in the region where the chicken endophyll was localized. Note that the orginal caudocranial orientation of the blastoderm is completely reversed under influence of the apposed quail Rauber's sickle fragment; bar: 1mm.

Fig. 15D. – Section through the caudal region of the chimera of Fig. 15C; SC: sickle canal; JE: junctional endoblast from quail; the arrowhead indicates transitional and/or sickle endoblast from quail; BI: chicken blood island in the lateral prolongation of chicken mesoblast (M); Feulgen staining; bar: 100μm.

observations are in agreement with the postulation of FLAMME (1989) that there exists an early morphogenetic endoblastic influence on extraembryonic mesoderm differentiation. However since the culture of quail-chick chimeras is limited in time we could not follow the final evolution of the endoblastic structures in the yolk sac. In the mouse, precursors of the extraembryonic mesoderm seem to be located in the caudolateral and caudal upper layer and are the earliest mesoderm to migrate through the streak (LAWSON et al., 1991). A surprising observation, in the mouse embryo, is that the erythropoeitic precursors of the yolk sac emerge earlier than the bulk of the vitelline endothelium, which is formed continuously throughout gastrula development (KINDER et al., 1999). Since, in the chicken embryo the mesoblast cells destined to become blood islands under influence of the junctional endoblast are localized most laterally in the area pellucida, we may presume that they also have ingressed through the primitive streak during early gastrulation. Indeed, through the most caudal part of the primitive streak (caudal node, containing part of the three germ layers) there occurs ingression of extraembryonic mesoderm (VAKAET, 1973). This occurs also in the middle region of the PS, according to GALLERA & NICOLET (1969). More cranial parts of the PS (without Hensen's node) give rise less frequently to extraembryonic mesoderm. In quail blastoderms developing on inverted egg yolk balls, cultured in egg white, the peripheral deep layer components (junctional endoblast and yolk endoblast) become locally necrotic or disappear wholly (CALLEBAUT et al., 2000b). This results in large defects in the formation of the area vasculosa and finally death of the embryo. In *Xenopus laevis* many models depict induction from the Spemann organizer (from which the descendants are notochord and head mesoderm) as a gradient of dorsalizing factors that diffuse across the marginal zone. The distance of a marginal zone cell from the Spemann organizer at gastrulation would then determine its dorsoventral identity. Thus the ventral blood islands, which were proposed to arise from tissue furthest away from the Spemann organizer were thought to be specified by the absence of organizer signaling. Indeed in explanted blastula-stage marginal zones a distance pattern develops with a restricted ventral blood island-forming region at the vegetal pole, that is independent of the patterning activity of Spemann's organizer (KUMANO et al., 1999). In molecular terms, dorsoventral patterning of the mesoderm is thought to result from antagonistic interaction between ventral mesoderm inducers as bone morphogenetic proteins (BMP) and their inhibitory binding proteins, including chordin, noggin and follistatin, which are produced by the Spemann organizer (GRAFF, 1997; THOMSEN, 1997). In the chicken blastoderm the blood islands also form in marginal tissue, furthest away from cranial structures such as notochord and head mesoderm. If we compare the localization of the blood-forming regions in the revised *Xenopus* blastula fate map (LANE & SMITH, 1999) with the localization of the first blood islands in the chicken blastoderm we see that they appear at the vegetal limit in the leading-edge mesoderm in both species.

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