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Rauber's sickle generates only extraembryonic tissues (junctional- and sickle endoblast), and, by positional information, organizes and dominates the whole avian blastoderm (gastrulation, neurulation and blood island formation)

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ABSTRACT. When the Rauber's sickle is (sub)totally mechanically removed from unincubated chicken blastoderms, their further evolution in culture is mostly disturbed. When,after removal of the chicken Rauber's sickle, a quail Rauber's sickle is placed isotopically, the normal development is totally restored. In such quail-chicken chimeras quail cells were found only in extraembryonic tissues, i.e. in junctional- and sickle endoblast and not among cells of the embryo proper (not in upper layer, nor in mesodermal, nor in endodermal cells). Further, we compared the inducing potencies of quail sickle endoblast placed on different regions of unincubated chicken blastoderms, either in the presence or absence of Rauber's sickle material. If a fragment of quail sickle endoblast was placed on the anti-sickle region of an unincubated chicken blastoderm from which the Rauber's sickle was (sub)totally removed, then often starting from this anti-sickle region an embryo presenting gastrulation and/or neurulation phenomena was induced but no blood islands were formed. So our study demonstrates that Rauber's sickle quantitatively and qualitatively dominates or inhibits ectopically-placed sickle endoblast. Earlier studies and the present study indicate the existence of a temporo-spatially bound cascade of gastrulation and neurulation phenomena and blood island formation in the avian blastoderm, starting from Rauber's sickle, the primary major organizer with inducing, inhibiting and dominating potencies.

KEY WORDS : avian blastoderm, Rauber's sickle, junctional endoblast, sickle endoblast, gastrulation, neurulation, blood island formation, positional information.

INTRODUCTION

Our knowledge about the developmental events and the inductory role and function of the different deep layer elements in avian blastoderms has improved only recently (Callebaut 1993a, b, c ; 1994 ; Callebaut & Van Nueten, 1994, 1995, 1996 ; Callebaut et al., 1999a ; Callebaut et al., 2000b). The different components of an unincubated quail blastoderm and surrounding structures are represented in Fig 1. The term Rauber's sickle (Rauber, 1876) is used instead of Koller's sickle (Koller, 1882), since RAUBER was the first to describe it (Callebaut & Van Nueten, 1994). The anti-sickle region 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 since neither endophyll, nor Rauber's sickle material is present (Callebaut & Van Nueten, 1995 ; Callebaut et al., 1998). 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 the caudal underlying peripheral subgerminal ooplasm without an underlying cavity. In the caudo-central region of the area centralis, a more or less developed sheet of endophyll can be seen. We use the term endophyll (Celestino Da Costa, 1948) and not endoblast or hypoblast to distinguish it from sickle endoblast (derived later by centripetal outgrowth from Rauber's sickle, Callebaut & Van Nueten, 1994). Endophyll cells and primordial germ cells all contain the same kind of deep central ooplasm (Callebaut & Vakaet : the so-called δ-ooplasm ; Callebaut, 1987) as the nucleus of Pander (Pander, 1817) and are originally localized in the surface of the latter, indicating a common origin (Callebaut, 1984). By contrast, Rauber's sickle cells and sickle endoblast contain more peripherally- and superficially-localized γ- and/or δ- ooplasm (Callebaut, 1987). Thus endophyll and sickle endoblast contain different ooplasms. Previous studies (Callebaut & Van Nueten, 1994 ; Cal-

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LEBAUT et al., 1996a, 1997a) indicated that a 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 center (NIEUWKOOP, 1969, 1973) in amphibian blastulas. Recently this has been confirmed by the study of goosecoid genes in avian blastoderms. Indeed LEMAIRE et al. (1997) found strong expression of one of the goosecoid genes (GSX) in the upper layer above Rauber's sickle, suggesting induction by the latter. So, goosecoid expression was not found in Rauber's sickle, but above. Later this upper layer will ingress and form the also-GSX-expressing primitive streak and Hensen's node. The latter are close to the amphibian organizer in SPEMANN & MANGOLD'S definition (1924). The junctional endoblast forms a whitish structure and can easily be seen at the surface of the living blastoderm *in*

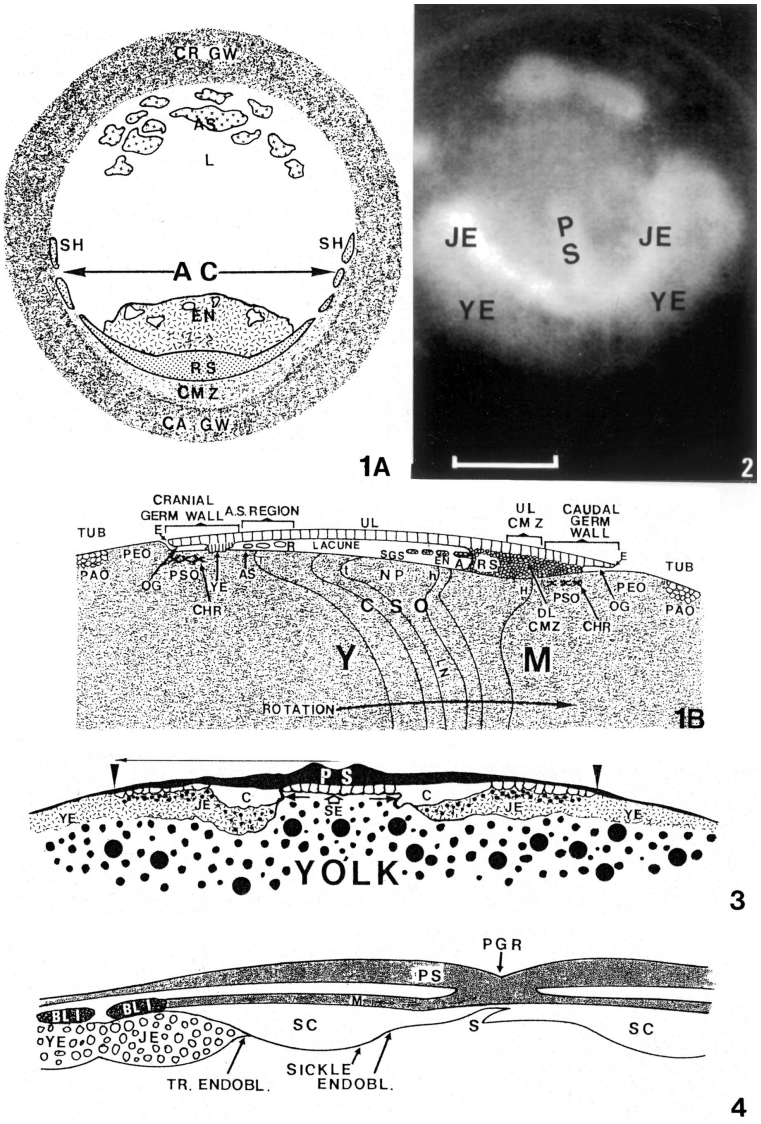


Fig. 1. – A. – Schematic representation of the components of the unincubated chicken blastoderm seen from below after removal of the subgerminal ooplasm, ready for *in vitro* culture. AC, area centralis ; AS, anti-sickle region ; CA GW, caudal germ wall ; CMZ, caudal marginal zone, more or less transparent ; CR GW, cranial germ wall ; EN, incomplete endophyll sheet ; L, lacune in the deep layer ; RS, Rauber's sickle and SH, fragmentary sickle horns enclosing the area centralis. B. – Schematic representation of a mediosagittal section through an unincubated quail blastoderm with surrounding ooplasms after fixation *in situ* on the egg yolk ball ; AS : anti-sickle ; CHR, chromosome clusters (CALLEBAUT, 1994) ; CSO, central subgerminal ooplasm ; DL CMZ, deeper part of the caudal marginal zone ; E, edge of the blastoderm ; EN, incomplete endophyll layer ; h, heel-shaped part of the nucleus of Pander ; 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, bended 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) ; NP, nucleus of Pander (1817) ; OG, early overgrowth zone (CALLEBAUT & MEEUSSEN, 1988) ; PAO, paragerminal ooplasm forming a tubulin (TUB) rich ring at distance from the edge of the blastoderm (CALLEBAUT et al., 1996b) ; PEO, perigerminal ooplasm ; PSO, peripheral subgerminal ooplasm ; RS, Rauber's sickle ; 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) ; t, toe-shaped part of the nucleus of Pander ; T : toe-shaped part of the surrounding yolk layers ; 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 ; UL, upper layer ; YE, early development of the yolk endoblast, growing into the peripheral subgerminal ooplasm ; 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.

Fig. 2. – Stereomicroscopic photomicrograph of a living quail embryo (Stage 2-3 of HAMBURGER & HAMILTON, 1951) *in situ* on its egg yolk ball, incubated for approximately 8-10h ; JE, junctional endoblast ; PS, primitive streak ; YE, yolk endoblast. Bar : 1mm.

Fig. 3. – Schematic drawing of a transverse section through the caudal part of the primitive streak region (localized between the two vertical arrowheads as a region where the 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 on the right indicate the transitional endoblast (CALLEBAUT & VAN NUETEN, 1994), which connects the sickle endoblast with the junctional endoblast (JE) ; (C), pararchenteric canals from which the sickle canal will develop. Between the lateral part of the junctional endoblast and the deep side of the primitive streak region, there are numerous extensions and small cavities, while between the more lateral yolk endoblast (YE) and the UL, there are no cavities and no extensions.

Fig. 4. – Schematic representation of the spatial relationship of the first formed blood islands (BL I) with the three germ layers in the caudal part of the chicken blastoderm after approximately one day incubation (stage 7-8 of HAMBURGER & HAMILTON, 1951). M : mesoblast ; PGR : primitive groove ; PS : primitive streak ; S : median septum formed by median sickle endoblast ; SC : sickle canal or sickle cavity localized above the sickle endoblast in the concavity of the sickle-shaped junctional endoblast (JE) ; YE : yolk endoblast.

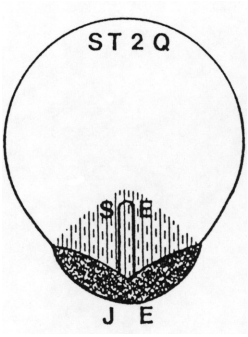


Fig. 5. – Schematic representation of the localization (corresponding to stage 2 of HAMBURGER & HAMILTON, 1951) of the two extraembryonic tissues, derived from Rauber's sickle : SE : quadrant-shaped layer of sickle endoblast extending from Rauber's sickle in a cranial and centripetal direction under the upper layer, below and laterally from the primitive streak (CALLEBAUT et al., 1997b) ; JE : V-shaped junctional endoblast derived from Rauber's sickle by local proliferation into the subgerminal ooplasm (CALLEBAUT & VAN NUETEN, 1994).

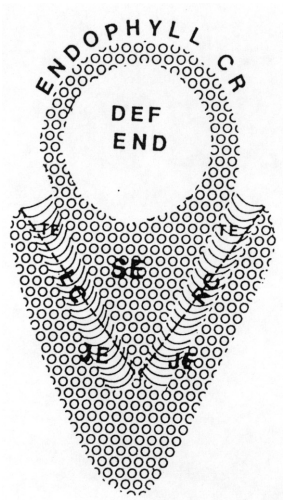


Fig. 6. – Schematic drawing of the anchor-shaped spreading (seen from dorsally after removal of the upper layer and primitive streak region at stage 3-4 of HAMBURGER & HAMILTON in the avian blastoderm) of the two cell lineages (sickle endoblast : SE and junctional endoblast : JE), derived from Rauber's sickle ; the small circles represent cellular connections between the sickle endoblast or junctional endoblast and the removed superficial layer ; LC : left part of the sickle canal ; RC : right part of the sickle canal ; TE : transitional endoblast ; DEF END : place where the definitive endoderm extends radially ; ENDOPHYLL CR : localization of the endophyllic crescent and wall (simplified after CALLEBAUT & VAN NUETEN, 1994).

situ on the egg yolk ball of a quail egg after approximately 9h incubation (Fig. 2). It forms the V- or U-shaped part of the deep layer (visible through the transparent upper layer) in the early avian primitive streak embryo. It is derived from Rauber's sickle cells that migrate into the neighbouring ooplasm during early incubation and has a typical histological aspect (CALLEBAUT & VAN NUETEN, 1994). The angle formed by the junctional endoblast is bisected by the primitive streak formed in the upper layer (Figs 2, 3). Laterally to the convexity of the junctional endoblast, one can distinguish the yolk endoblast (developing in the caudal marginal zone), which has a less dense, white aspect but is more voluminous than the junctional endoblast. 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., 2000c). In a recent study (CALLEBAUT et al., 2000a), we observed that during avian neurogastrulation, a sickle-shaped canal (called "sickle canal") develops parallel with/and in the concavity of the U-shaped junctional endoblast by caudal fusion of the pararchenteric canals (Figs 3, 4). We observed the existence of a spatial relationship between the sickle canal or sickle cavity (limited below by sickle endoblast with associated junctional endoblast) and the early appearance of blood islands (Fig. 4). We used the term sickle endoblast because we have demonstrated, by using the quail-chick chimaera technique, that this part of the deep layer is directly derived from Rauber's sickle by centripetal and cranial growth and/or migration (CALLEBAUT & VAN NUETEN, 1994 ; CALLEBAUT et al., 1997b) (Figs 5,6). When a Rauber's sickle fragment is placed ectopically on any region of an isolated central part of the area centralis, then a primitive streak, a neural plate (CALLEBAUT et al., 1997a) and blood islands (CALLEBAUT et al., 2000a) are always induced after culture *in vitro*. Recently, we have shown that Rauber's sickle or later junctional endoblast is indispensable for the development of blood islands in the avian blastoderm (CALLEBAUT et al., 2002). A recent experimental *in vitro* study (CALLEBAUT et al., 2003a) demonstrated that a primitive streak (PS) in avian blastoderms is induced by diffusion of signalling molecules emanating from Rauber's sickle according to the basic concept of "positional information" (WOLPERT, 1969, 1981). Indeed, even without direct contact between a quail Rauber's sickle and the adjacent reacting upper layer (by interposition of a vitelline membrane), a primitive streak can be induced in the isolated area centralis or anti-sickle region of unincubated chicken blastoderms. From recent molecular biology studies (KNOETGEN et al., 1999a, b ; FOLEY et al., 2000 ; KNEZEVIC and MACKEM, 2001) it was concluded that the deep layer (containing sickle endoblast) elicits no definitive changes in the adjacent host upper layer after transplantation. However, these authors have not taken into account the fact that Rauber's sickle was always present in their experimental set-up and thus could have an inhibiting effect. Indeed in a recent study (CALLEBAUT et al., 2003b) we observed that if a quail endoblast fragment was placed on the isolated central Rauber's sickle-free part of a chicken area centralis, in culture, then a definitive primitive streak and neural plate were induced. This is confirmed by the present experimental study, since in a whole unincubated chicken blastoderm from which the Rauber's sickle has been (sub)totally removed, a fragment of sickle endoblast can also induce an embryo whilst this

induction is totally suppressed when an intact Rauber's sickle is still present. This indicates that Rauber's sickle dominates and can abolish totally the inducing activity of sickle endoblast. If no Rauber's sickle is present, then sickle endoblast can take over the function of Rauber's sickle, but without blood island formation. Sickle endoblast can thus be considered as a secondary, minor organizer of the avian blastoderm, normally dominated by Rauber's sickle, the primary major organizer.

MATERIAL AND METHODS

We used unincubated chicken (*Gallus domesticus*) blastoderms (or parts of) and sickle endoblast from 7-8h *in ovo* incubated quail (*Coturnix coturnix japonica*) blastoderms. Storage of the unincubated eggs for 1-2 days at room temperature seemed to increase the visibility of the Rauber's sickle. This is probably due to its increase in volume since we observed uptake of voluminous subgerminal "yolk islands" by encircling extensions of Rauber's sickle cells (Callebaut, 1994) and premitotic DNA synthesis in Rauber's sickle cells, even at room temperature (Callebaut, 1989). Stages of embryonic development were indicated according to Hamburger & Hamilton (1951) as (HH) or according to Eyal-Giladi & Kochav (1976) as (EK). After opening of the chicken eggs and removal of the egg white, the egg yolk balls were placed in Ringer's solution. The vitelline membrane was sectioned all around the equator of the yolk ball and removed from the yolk by a slow movement in the direction of/and bending 180˚ over the germ. In this manner, unincubated chicken blastoderms still adhering to the vitelline membrane and underlying subgerminal ooplasm, could usually be separated from the yolk. Normally the required quail sickle endoblast stage, corresponding to Stage 2-3 of Hamburger & Hamilton (1951) in the chicken, is reached in the *in toto* incubated quail egg after approximately 7-8h incubation. This is shorter than for *in toto* incubated chicken eggs, since the latter are approximately six times larger and the time to warm up lasts longer. First, the quail yolk balls are inspected in Ringer solution under a stereomicroscope in order to observe the external morphology of the germ disc. When a short primitive streak and the "legs" of the V-shaped junctional endoblast are visible from the surface, as seen on Figs 2 and 5, the quail germ disc can be used for excision of the sickle endoblast. Each experimental procedure used is described in the legends of the figures or in the text of the results or is represented in a scheme accompanying the microphotographs of the associated blastoderm parts in culture. The blastoderms were cultured according to the technique of Spratt (1947). This semi-solid culture medium allowed microsurgery and further culture on the same substratum. Instead of Petri dishes, the culture vessels described by Gaillard (1949), on which an optical flat glass cover was sealed with hot paraffin, were used. Stereomicroscopic Polaroid photographs were taken in the same direction at the beginning, during and at the end of the culture period (23-32h). Fixation was performed overnight in a modified Heidenhain's fixative (Romeis, 1948) containing 0.5 g sodium chloride, 2 g trichloracetic acid, 4 ml acetic acid, 20 ml formalin and 80 ml water. After rinsing in tap water, the blastoderms were stained *in toto* with Unna to enable their orientation in the paraffin wax to be seen. After rapid dehydration in a graded series of alcohol and embedding in paraffin, 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), to enable identification of the origin of the nuclei. This allowed us to observe the typical central or subcentral chromatin granule (most obvious with low power objectives) of the grafted quail cells (Callebaut 1968 ; Koshida & Kosin, 1968 ; Le Douarin & Barq, 1969) as well as to observe their relation with the chicken tissue. In the photographs of the sections, the associated blastoderm parts are represented with their deep side and sickle endoblast material directed upwards, since they were cultured in this orientation.

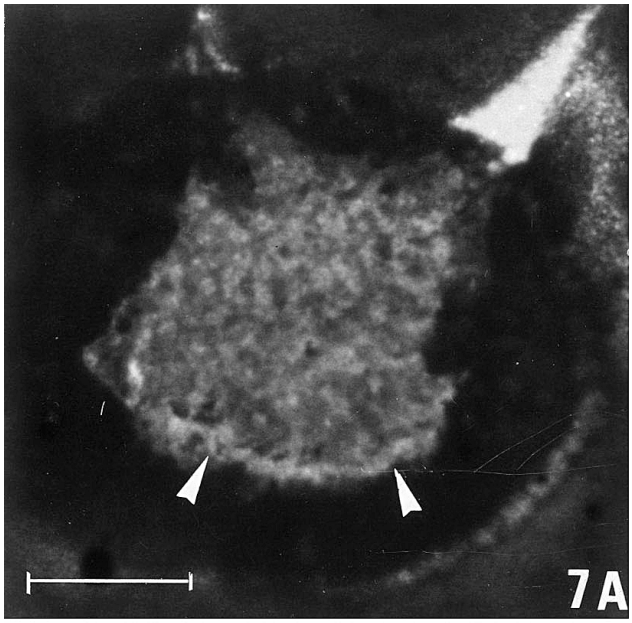
RESULTS

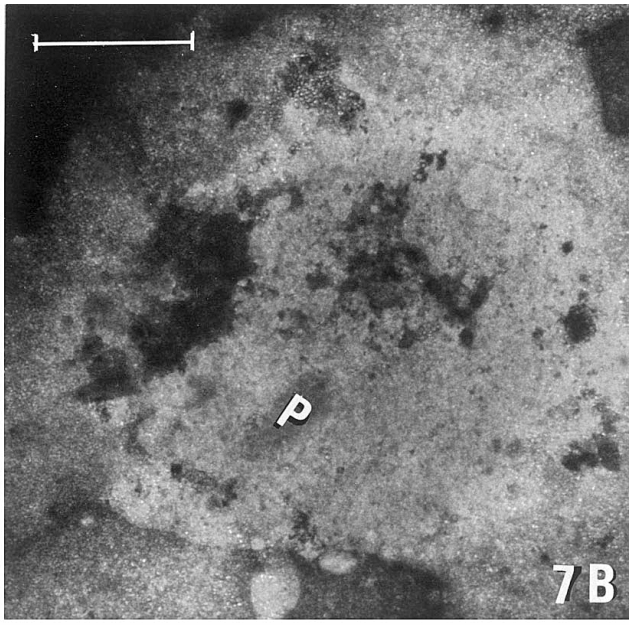
(Sub)total removal of Rauber's sickle (n=15)

After mechanical removal of the Rauber's sickle from an unincubated chicken blastoderm (Fig. 7A) and culture, in about 30% of the cases no complete embryonic development was observed. Sometimes only a preneural plate was formed without primitive streak. The upper layer expanded considerably over the culture medium. In the remainder of cases a small primitive streak with centripetally-directed axis (in the direction of the endophyll), appeared (Fig. 7B). This primitive streak started from a point where formerly the circumference of the autochthonous Rauber's sickle was observed (often from a sickle horn region, since there the Rauber's sickle material is barely visible and difficult to remove). After prolonged culture, a both a primitive streak and a neural plate (confirmed on sections) could be observed (Fig. 7C). Obviously, the development was slower than normal.

Isotopic exchange of a Rauber's sickle from unincubated chicken blastoderms with a quail Rauber's sickle (n=10)

From unincubated chicken blastoderms, the autochthonous Rauber's sickle, was removed. Then a quail Rauber's sickle (also from an unincubated quail blastoderm) was apposed isotopically on this chicken blastoderm (Fig. 8A). Already after 8h of culture a normally-developed primitive streak was observed, starting from the place where the quail Rauber's sickle was placed (Fig. 8B). After approximately 32h of culture, a completely normal embryo corresponding to stage 9 of Hamburger & Hamilton (1951) was seen. On sections through such chimeras quail cells were found only in the form of quail sickle endoblast or junctional endoblast. In the caudal part of the blastoderm, the quail sickle endoblast lines the sickle canal (Fig. 8C). The quail junctional endoblast is found close to the forming blood islands on which it has an inducing effect (Callebaut et al., 2000a, 2002). In the cranial part of the chimeric blastoderm, quail sickle endoblast is found cranially and laterally from the radially expanding definitive chicken endoderm (as was already observed after ectopic placement of a quail Rauber's sickle : Callebaut & Van Nueten, 1994) (Fig. 6). In the remainder of the blastoderm no quail cells were found (not in the superficial layer, nor in the chordomesoblast, nor in the prechordal plate, nor in the definitive gut endoderm) (Fig. 8D).





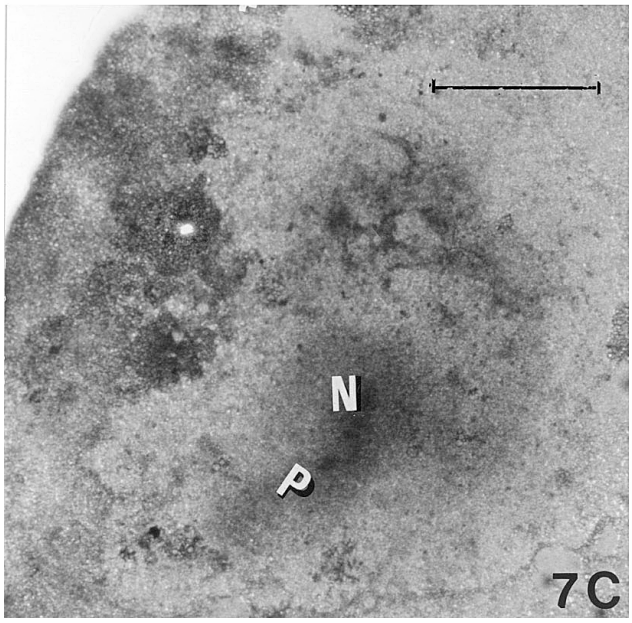


Fig. 7A. – Stereomicroscopic view from the lower side of an unincubated chicken blastoderm from which Rauber's sickle was mechanically removed (indicated by two arrowheads) at the start of the culture period ; bar : 1 mm.

Fig. 7B. – Stereomicroscopic view of the same blastoderm as seen in Fig 7A, after 22h of culture : a small primitive streak (P) has developed from laterally close to the sickle horn region ; bar : 1 mm.

Fig. 7C. – The same blastoderm as in Fig. 7B after 26h of culture before fixation ; besides a primitive streak (P), a neural plate (N) is also visible ; bar : 1 mm.

Placement of a fragment of quail sickle endoblast on the isolated (Rauber's sickle-free) anti-sickle of an unincubated chicken blastoderm (n=12) (Fig. 9A)

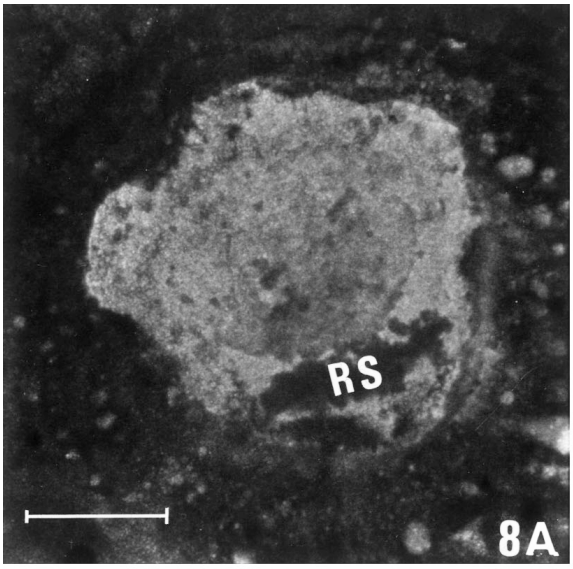
A (pre)neural plate always developed after culture (often with a shallow median neural groove) in the thickened upper layer, adjacent to the quail sickle endoblast (Fig. 9B). No ingression phenomena and no primitive streak were observed.

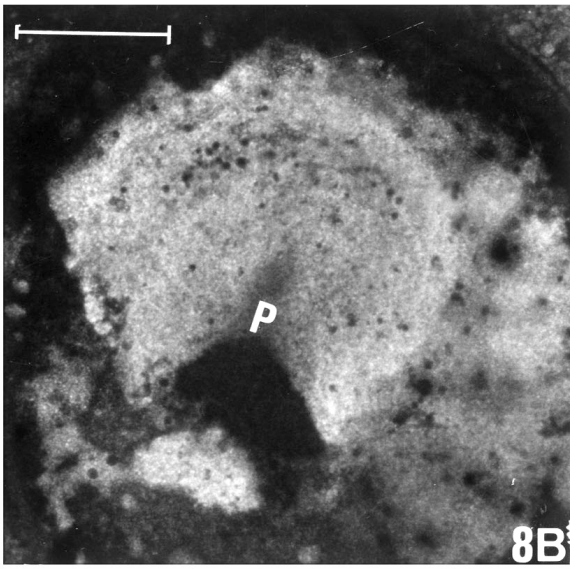
Placement of a fragment of quail sickle endoblast on the anti-sickle region of an intact unincubated chicken blastoderm (n=8)

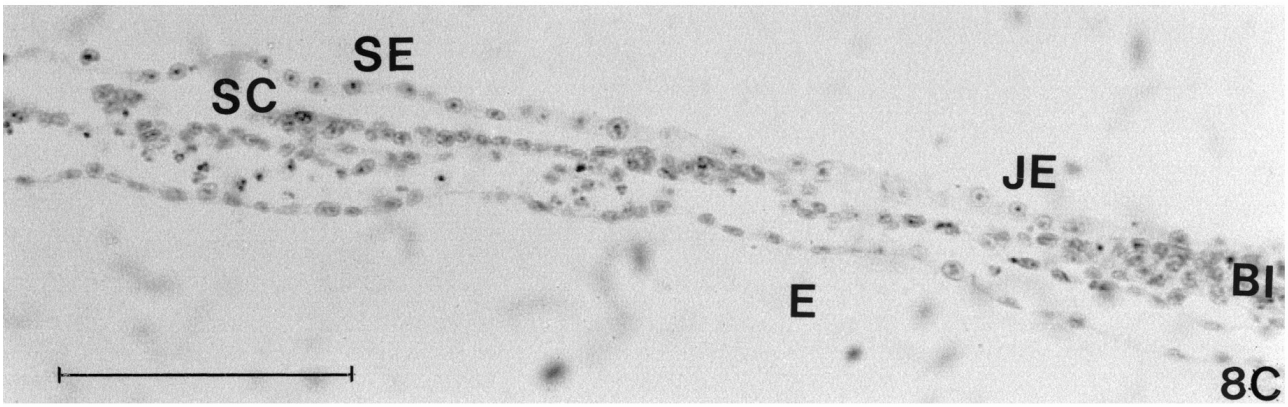
During culture, the quail sickle endoblast rapidly became invisible under the stereomicroscope and no induction phenomena were ever observed. On sections no thickening of the upper layer adjacent to the apposed quail sickle endoblast was seen. Here, clearly neither gastrulation nor neurulation phenomena took place. Also no blood island formation occurred in relation to the apposed sickle endoblast.

Placement of a fragment of quail sickle endoblast on the anti-sickle region of an unincubated chicken blastoderm from which the autochthonous Rauber's sickle was (sub)totally removed (Fig. 10A) (n=20)

After culture, in about half of the cases, a centripetally-oriented embryo was induced in the anti-sickle region by the quail sickle endoblast (independent from its original polarity). When no accessory small embryo developed from the region where the autochthonous Rauber's sickle was removed, then the induced embryo progressively extended over the whole area centralis (Fig. 10B) and, besides a primitive streak, a large neural plate was seen. On sections the quail sickle endoblast was seen medially under the primitive streak region (Figs 10C and C'). Sections







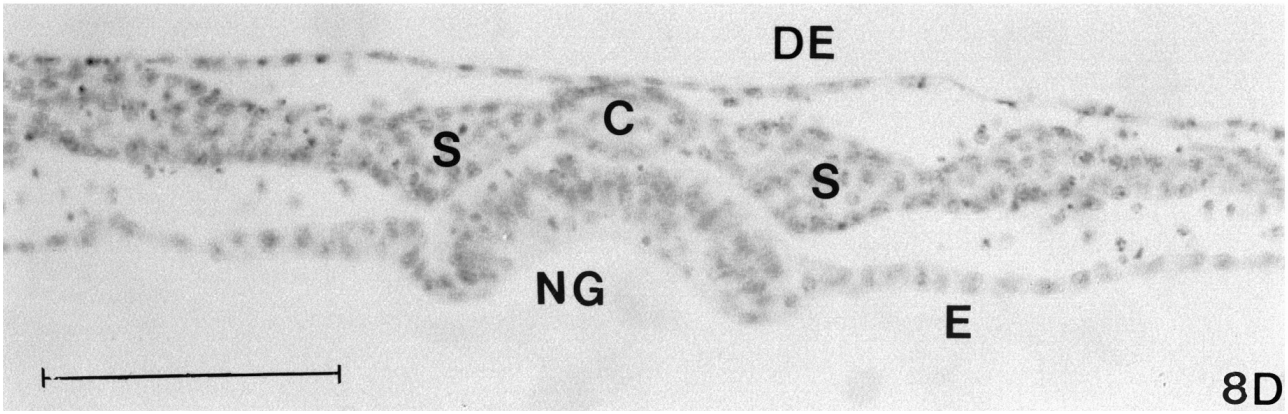


Fig. 8A. - At the start of the culture : a quail Rauber's sickle (RS) was isotopically placed where the autochthonous chicken Rauber's sickle was removed ; bar : 1 mm.

Fig. 8B. – The same chimera as seen in Fig. 8A after 8h of culture : a completely normal blastoderm with primitive streak (P) develops ; bar : 1 mm.

Fig. 8C. – Section through the caudal region of the same embryo after 32h of culture ; SE : quail sickle endoblast ; JE : quail junctional endoblast in the immediate neighborhood of a blood island (BI) ; SC : sickle canal or sickle cavity ; E : epiblast ; Feulgen staining ; bar : 100 μm.

Fig. 8D. – Section through the more cranial part of the same embryo ; DE : definitive endoderm ; C : chorda ; S : somites ; NG : neural groove ; E : epiblast ; no quail cells are visible ; Feulgen staining ; bar : 100 μm.

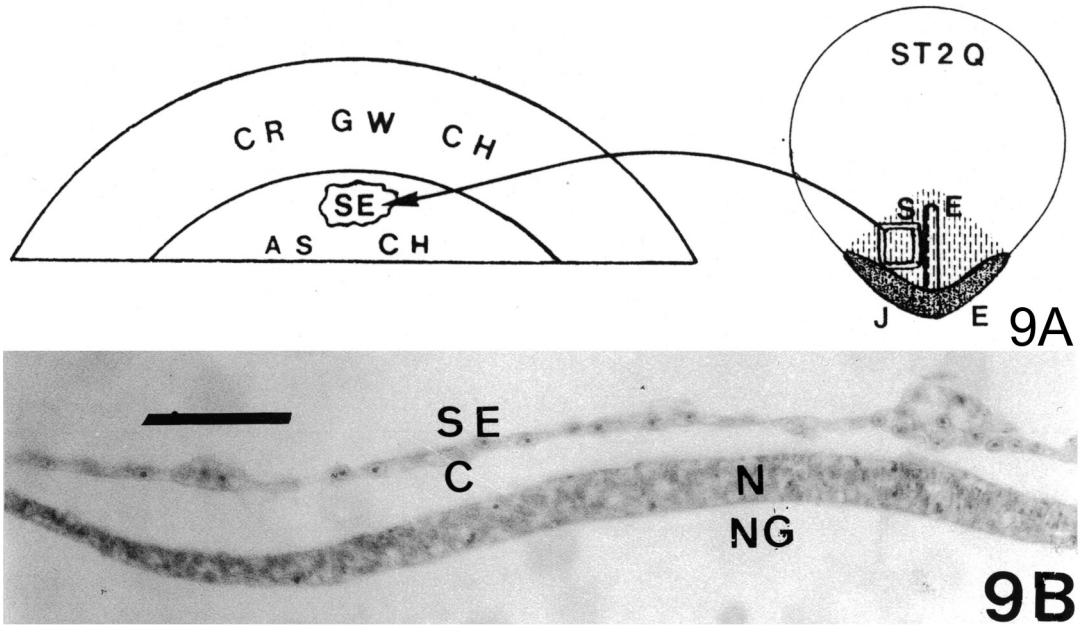
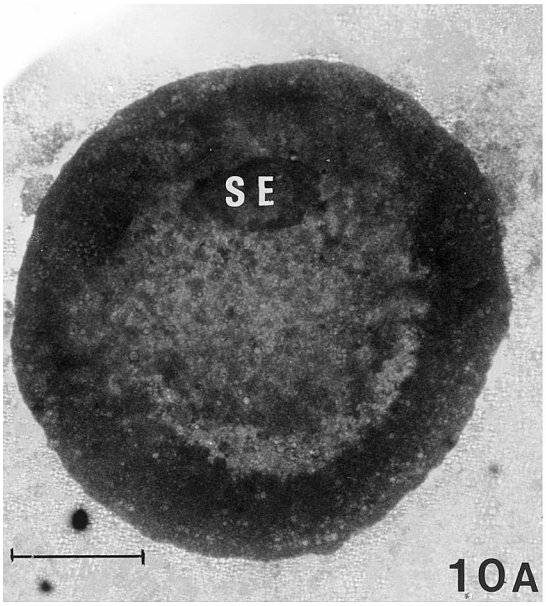


Fig. 9A. – Schematic drawing representing the excision of a fragment of quail sickle endoblast (SE) (at the right) and its transplantation (indicated by a long curved arrow) on the deep side of the anti-sickle region of an unincubated chicken blastoderm (ASCH) ; CR GW CH : cranial germ wall of chicken.

Fig. 9B. – Section through a chimera composed as represented in Fig. 9A, after 24h of culture : the (pre)neural thickening (N) of the upper layer and the parallel inducing quail sickle endoblast layer (SE) have approximately the same extent and are separated by a cavity (C) ; no ingression phenomena are seen ; a broad neural groove (NG) is visible ; Feulgen staining ; bar : 100 µm.



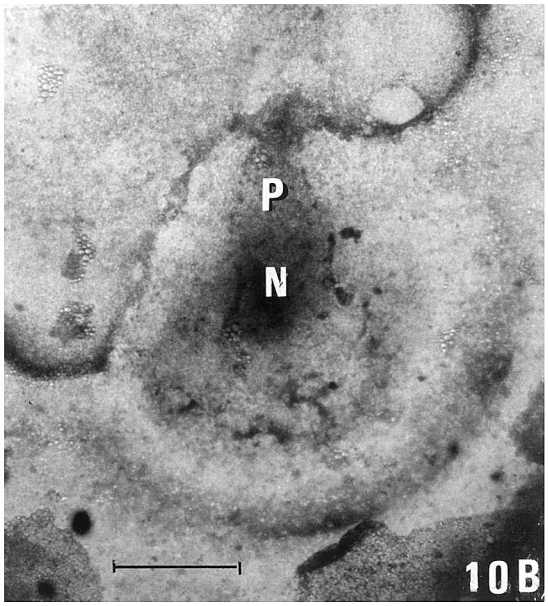


Fig. 10A. – Stereomicroscopic view of the deep side of an unincubated chicken blastoderm from which the Rauber's sickle was removed and on which a fragment of quail sickle endoblast (SE) was placed over the anti-sickle region ; bar : 1 mm.

Fig. 10B. – Stereomicroscopic view of the same chimera as seen in Fig 10A after 26h of culture, before fixation ; an embryo extending from the anti-sickle region over the whole area centralis has been induced by the apposed quail sickle endoblast ; P : primitive streak ; N : neural plate ; no accessory embryo has developed from the region of the removed chicken Rauber's sickle, indicating a successful total removal ; bar : 1 mm.

at the level of HENSEN's node (most cranial part of the primitive streak) revealed the presence of quail sickle endoblast on both sides, separated by the median, radially-expanding definitive endoderm (Figs 10 D and D'). More cranially, the quail sickle endoblast was found medially adjacent to/but separated by a narrow space from the (pre)neural plate (Figs 10 E and E'). No junctional endoblast and no blood islands were observed in the original anti-sickle region. This indicates that sickle endoblast cannot give rise to junctional endoblast even after more than

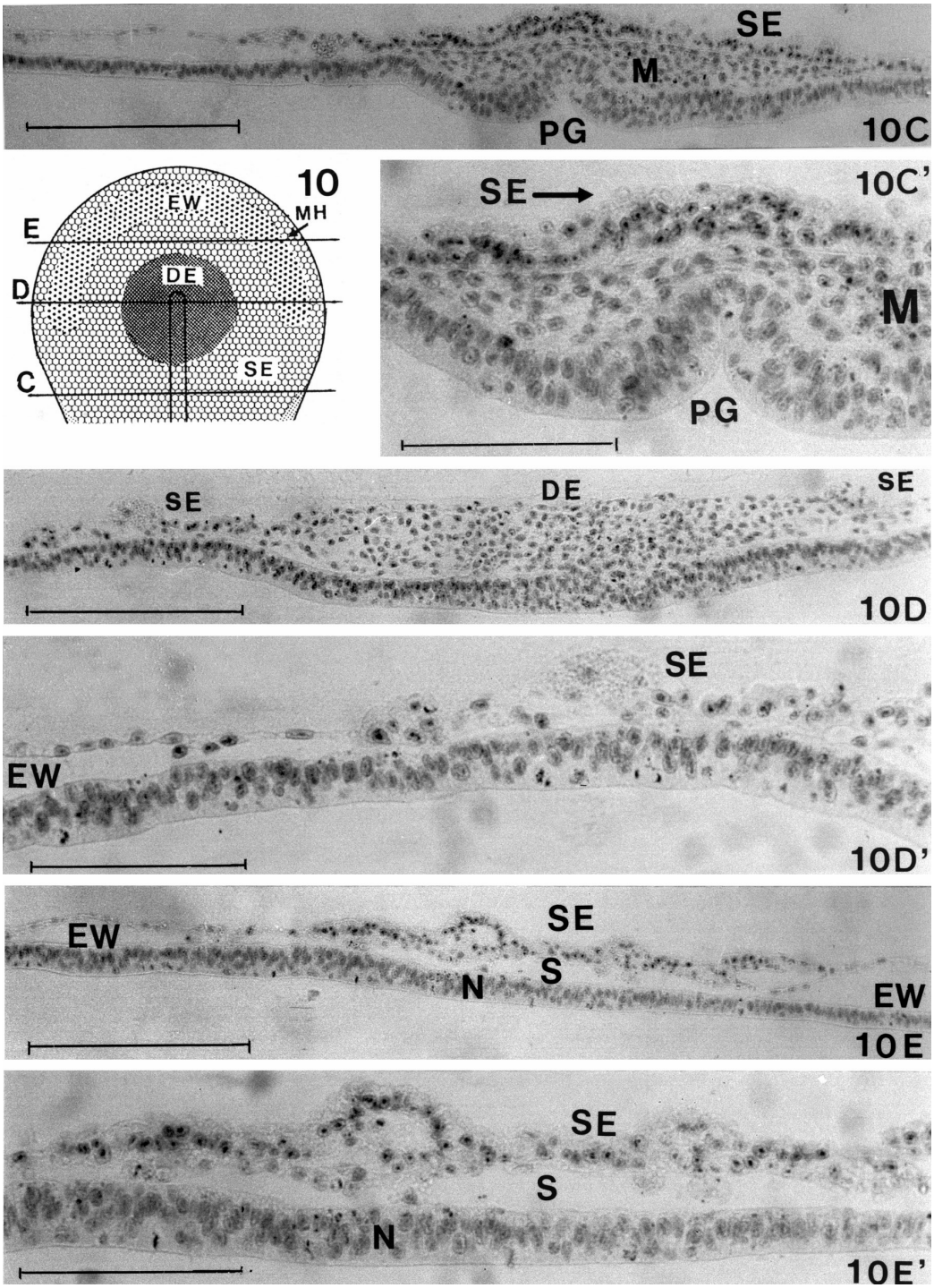


Fig. 10. – Schematic drawing representing the different deep layer components found in the definitive cranial part of the blastoderm of Fig. 10B ; EW : endophyll wall ; MH : marginal hypoblast ; SE : sickle endoblast ; DE : definitive endoderm expanding radially around the top of the primitive streak (Hensen's node) ; the horizontal lines C, D and E indicate the level of the sections seen respectively in Figs 10C and 10C' ; Figs 10D and 10D', Figs 10E and 10E'.

Fig. 10C. – Section through the primitive streak region of the chimera of Fig. 10B at the level C as indicated on the schematic drawing ; quail sickle endoblast cells (SE) are seen in the median region above the induced primitive streak and primitive groove (PG) ; M : mesoblast formed by ingression via the primitive streak ; Feulgen staining ; bar : 200 µm.

Fig. 10C' – The same section as in Fig. 10C at a higher magnification with the same indications ; bar : 100 µm.

Fig. 10D. – Section through the nodus region of the chimera of Fig. 10B (at the level D as indicated on the schematic drawing) ; DE : definitive endoderm pushing the quail sickle endoblast (SE) radially to the periphery (visible on both sides) ; Feulgen staining ; bar : 200 µm.

Fig. 10D'. – Enlarged view from part of Fig. 10D ; SE : quail sickle endoblast ; EW : endophyll wall covered by chicken endophyll ; bar : 100 µm.

Fig. 10E. – More cranial section through the region of the neural plate *Anlage* (at the level E, as indicated on the schematic drawing) ; SE : median quail sickle endoblast separated from the neural plate (N) by a space (S) ; EW : endophyll wall on both sides, covered with chicken endophyll ; Feulgen staining ; bar : 200 µm.

Fig. 10E' – Part of section of Fig. 10E at a higher magnification with the same indications ; bar : 100 µm.

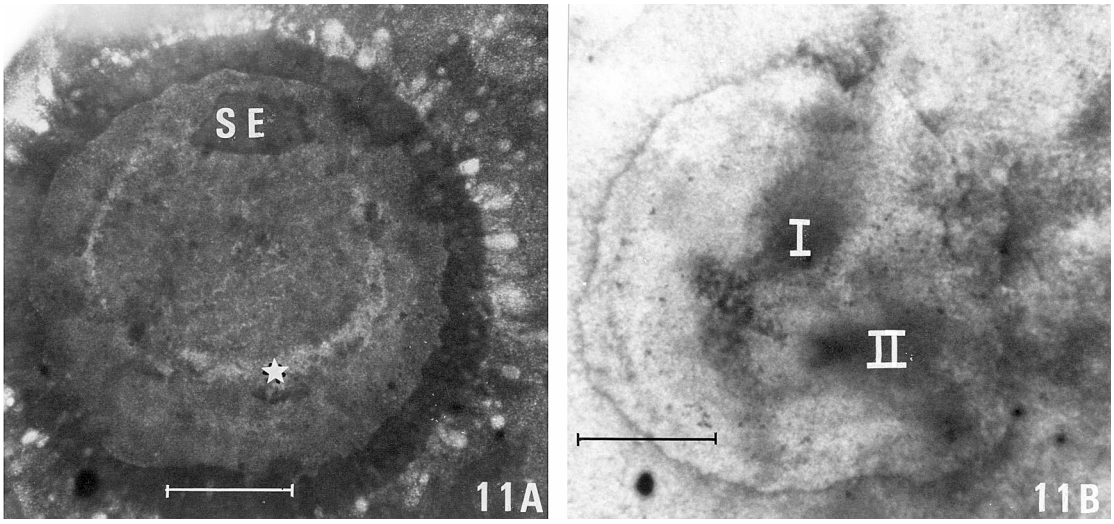


Fig. 11A. – Stereomicroscopic view of a chicken blastoderm from which the Rauber's sickle was mechanically removed (*) and on which a fragment of quail sickle endoblast (SE) was placed on the anti-sickle region ; bar : 1 mm.
Fig. 11B. – The same chimera as seen in Fig 11A, after 25h of culture : two centrally-oriented embryos (with a head against head localization) have formed in the blastoderm ; one (I) is induced by the apposed quail sickle endoblast fragment ; the other (II) is formed in the neighbourhood of the left sickle horn region ; bar : 1 mm.

one day of culture. This experiment also indicates that the caudal marginal zone (which remains present) in the absence of Rauber's sickle material is, on its own, not able to induce a primitive streak nor to inhibit the inducing effect of sickle endoblast apposed on the anti-sickle region. If, after removal of the Rauber's sickle and placement of a fragment of quail sickle endoblast on the anti-sickle region (Fig. 11A), only a small accessory embryo developed from part of the circumference where the autochthonous Rauber's sickle was originally localized before removal, then usually also an embryo appeared under inductory influence of the apposed quail sickle endoblast fragment (Fig. 11B). This embryo also presented a centripetally-directed axis, (independent from the original polarity of the sickle endoblast) with its head region against the head region of the accessory embryo. Both seem to compete for space in the area centralis as is also the case after grafting an ectopic Rauber's sickle on the anti-sickle region (Callebaut & Van Nueten, 1994). However, no quail junctional endoblast and no blood islands were formed in the embryo induced by the sickle endoblast. If a fully developed embryo developed, starting from the sickle-shaped region where Rauber's sickle was (sub)totally removed, then no embryo induction by the apposed quail sickle endoblast was observed. In these cases, on sections, no thickening of the upper layer adjacent to the sickle endoblast was seen, indicating the absence of gastrulation and/or neurulation phenomena. However in some cases only a limited preneurulation with space formation between the thickened upper layer and the quail sickle endoblast was seen. That the chicken Rauber's sickle material was removed incompletely could usually best be seen after culture for 24h or more. Indeed in these cases often a sickle-shaped area vasculosa (visible alive) appeared in the caudal part of the blastoderm behind the chicken embryo proper. On sections, blood islands were then found in the immediate neighborhood of some chicken junctional endoblast, derived from the remnants of the Rauber's sickle. We can conclude that if Rauber's sickle activity is strongly reduced, then sickle endoblast can still induce gastrulation and neurulation phenomena. If Rauber's sickle activity is totally absent, then the inducing power of the sickle endoblast material becomes maximal. If Rauber's sickle is fully present in a blastoderm, then the inducing activity of the sickle endoblast, placed on the anti-sickle region, will always be totally suppressed.

Placement of a fragment of quail sickle endoblast on the area centralis of an intact unincubated chicken blastoderm (Fig. 12A) (n=7)

After culture, the development was always seen to be much slower than normal. Sometimes two embryonic areas were found aligned (Fig. 12B) with a separate primitive streak in each area. In sections, in only one of the areas, the apposed quail sickle endoblast sheet was found.

DISCUSSION

In the past the very existence in unincubated avian blastoderms of a Rauber's sickle (1876) (formely called Koller's sickle : 1882) has been vigourously debated, for decades. Principal opponents to its real existence have been Peter (1938) and Pasteels (1937 ; 1940) who consigned the sickle to oblivion. By their strong scientific impact the sickle has been voluntarily or involuntarily forgotten by most investigators until the 1970s. So the Rauber's (Koller's) sickle was not even mentioned in the detailed description of experiments with avian blastoderms (Bellairs, 1971). The cleavage experiments of avian germ discs *in ovo* (Lutz, 1964) already suggested a functional relationship between the localization of Rauber's sickle fragments and caudocephalic axis formation. Vakaet (1962) concluded that in unincubated chicken blastoderms at least some indication of the existence of a Rauber's sickle was to be found. The reason for the doubt about the real existence of a Rauber's sickle in every unincubated avian blastoderm seems to be that it was only vis-

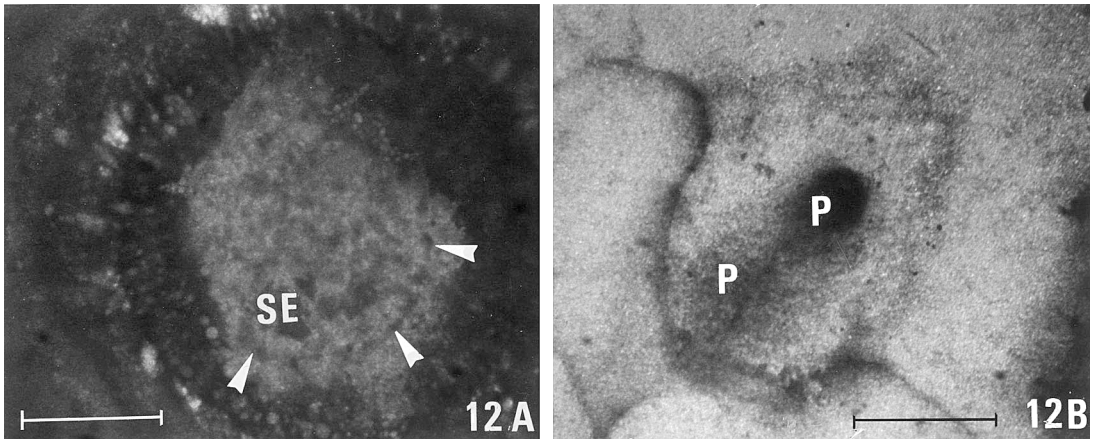


Fig. 12A. – On the deep side of the caudal part of the area centralis of a complete unincubated chicken blastoderm, presenting a narrow fragmentary Rauber's sickle (indicated by arrowheads), a fragment of quail sickle endoblast (SE) was placed at the start of the culture ; bar : 1 mm.
Fig. 12B. – The same chimera as seen in Fig 12A after 27h of culture : the development is much slower than normal ; P and P are two areas with separate aligned primitive streaks (confirmed by sectioning) ; bar : 1 mm.

ible from the surface of the freshly laid quail egg in about 30% of cases (Lutz, 1964 ; Fargeix, 1964). This can be improved with intraoocytal trypan blue labelling to 60% in the quail (Callebaut et al., 1998b). Also the detaching of the unincubated chicken blastoderm together with its covering vitelline membrane from the egg yolk ball can give problems. Indeed it then happened often that Rauber's sickle wholly or partially remained fixed to the subgerminal ooplasm and so was not observed. That a Rauber's sickle is present in nearly every blastoderm of unincubated chicken or quail eggs can be shown in sections (performed perpendicularly to the chalazal rotation axis) through the germ discs (after fixation *in toto* on their egg yolk balls). Indeed in sections after such a fixation, the future caudal part of the germ (with the Rauber's sickle adhering to the subgerminal ooplasm) and the future cranial part of the germ (with the anti-sickle region disrupted from the subgerminal ooplasm) can be easily recognized (Callebaut et al., 1998a). Because during several decades, Rauber's sickle was not considered as a deep layer component distinctly separable from the caudal marginal zone, its function remained unknown, until Callebaut & Van Nueten (1994) first systematically isolated and transplanted (without surrounding tissues) quail Rauber's sickles (which are usually much more voluminous than chicken Rauber's sickles). Thus their powerful inductive activity for the formation of mesoblast and definitive endoderm was shown. The structures and involved early induction phenomena in the avian germ disc present much homology with similar structures and phenomena taking place in the zebrafish egg (*Brachydanio rerio*) (Callebaut et al., 1996b). Recently Koos & Ho (1998) have shown that at the onset of gastrulation nieuwkoid expression becomes localized in a restricted region of the extraembryonic yolk syncytial layer, directly underlying the future dorsal shield. This part of the yolk syncytial layer in teleleosts seems thus to correspond to the Nieuwkoop center in the *Xenopus* embryo and to Rauber's sickle in avian germs. We observed that the tissues in the immediate neighborhood of Rauber's sickle (both the upper and deep part of the caudal marginal zone) have no inductive activity on their own (Callebaut et al., 1998a). In the present study we show once again that it is Rauber's sickle and not the caudal marginal zone that is able to induce a primitive streak, since after mechanical removal of the Rauber's sickle the caudal marginal zone was left intact *in situ*. This seems to be in contradiction with the conclusions of Bachvarova et al. (1998) and Bachvarova (1999). In their study a quail caudal marginal zone was associated *in vitro* culture with a chick cranial half blastoderm. In such associations often a centrally-directed primitive streak was observed starting from the cranial part of this cranial half. However we have shown that this also often occurs spontaneously in the absence of caudal marginal zone material (Callebaut & Van Nueten, 1993) by the presence of far cranially-extending, unrecognized sickle horn material in the cranial blastoderm half. Recently the developmental role of Rauber's sickle became still more fascinating with the discovery that after an initial inducing effect on mesoblast and definitive endoderm formation in the upper layer of the area centralis, it also induces blood island formation in the mesoblast that migrates peripherally over the junctional endoblast, directly below the flat epiblast, in the direction of the area opaca (Callebaut et al., 2000a). Moreover, we found that Rauber's sickle or Rauber's sickle-derived junctional endoblast was indispensable for early formation of blood islands (Callebaut et al., 2002). Although Rauber's sickle and the Rauber's sickle-derived sickle endoblast and junctional endoblast have a very important and indispensable inductive function for the development of the embryonic tissues during gastrulation and neurulation, our study demonstrates that they never give rise to cells of the embryo proper and therefore belong to the so-called extraembryonic part of the blastoderm (as is also the case for the Nieuwkoop center (Guger & Gumbiner, 1995). This is in contrast to the conclusion of the study of Izpisua-Belmonte et al (1993). Indeed, a problem in their experiments was that they could not isolate Rauber's sickle completely neither from the upper layer nor from the deep layer. So, always part of a region in the neighborhood of Rauber's sickle was also taken. The same holds true for their DiI applications to the very narrow and fragile chicken Rauber's sickles after which not only Rauber's sickle cells were labelled, but also upper layer cells, by inevitable leakage of

this stain. In conclusion, the avian Rauber's sickle fulfils the major postulate for homology with a functional Nieuwkoop centre, namely the potential for organizer induction without itself contributing to the new structure. Our present study once again demonstrates the value of the anti-sickle region of unincubated blastoderms as an experimental tool for demonstrating induction phenomena.

In the present study we show that sickle endoblast, if withdrawn from the influence of Rauber's sickle, has gastrulation and/or neurulation inducing potencies on the upper layer of the avian unincubated blastoderm, but it has no influence on blood island formation. Obviously, it also plays a role during normal development in the early organization (primitive streak formation) of the avian blastoderm in combination with its mother tissue, Rauber's sickle. So, the area where a primitive streak-inducing action can take place is considerably extended during early incubation from a sickle shape (Rauber's sickle) to approximately the whole caudal quadrant of the blastoderm (Fig 5), i.e. the area where the sickle endoblast penetrates into the endophyll (Callebaut et al., 1997b). During the first hours of culture, we observed in the latter study that an intimate contact developed (before a real primitive streak with ingressing upper layer cells appears) between the infolded thickened chicken upper layer and the quail sickle endoblast on the midline. This suggested already a primary influence of sickle endoblast on the formation of the primitive groove and primitive streak. These earlier observations can probably been explained as the result of the here described induction phenomena by fragments of quail sickle endoblast. Indeed, our present experiments, where a fragment of sickle endoblast was placed on the area centralis of an intact (Rauber's sickle-containing) unincubated blastoderm, seem to indicate that during early culture a temporary competition takes place between the sickle endoblast and the Rauber's sickle. But there also, Rauber's sickle finally overshadows the early effect of the sickle endoblast. That in the present experiment no junctional endoblast developed from the grafted sickle endoblast seems to indicate that the differentiation of Rauber's sickle material into sickle endoblast is irreversible and that only Rauber's sickle versus junctional endoblast can induce blood islands. The homeobox gene CHex is expressed in Rauber's sickle and sickle endoblast (Yatskievych et al., 1999). CHex transcripts were also detected within blood islands beginning at stage 4 and in extraembryonic and intraembryonic vascular endothelial cells. Since we have shown that Rauber's sickle and junctional endoblast have an inducing effect on blood island formation, we can postulate an unknown relationship with the CHexgene.

The molecular basis of neural induction has been extensively studied in *Xenopus laevis*, and it was found to be tightly coupled to the establishment of the dorso-ventral axis (De Robertis & Sasai, 1996 ; Hemmati-Brivanlou & Melton, 1997). In frogs, the prospective ectoderm is induced by bone morphogenetic proteins (BMPs). In contrast, a neural development requires the inactivation of BMPs and is achieved by direct complex formation between BMPs and neural inducing factors such as chordin, noggin or follistatin (Piccolo et al., 1996 ; Zimmermann et al 1996). In the chick blastoderm at early stages, the prospective epidermis is characterized by the expression of the homeobox gene DLX5, which remains an epidermal marker during gastrulation and neurulation and enables it to be distinguished from the more central neural plate (PERA et al., 1999). That vertical signals from the lower layer are necessary for the establishment of the neural plate has been shown by the latter authors by repeated extirpations of the underlying endoblast. In the absence of the lower germ layers, the epidermis expanded into the region that normally forms the neural plate. Knoetgen et al. (1999a) analysed the GANF (Gallus anterior neural fold) – inducing potential of various tissues at different stages during chick development by transplantation to the outer margin of the area pellucida, where the epiblast cells are fated to become epidermis (Spratt, 1952 ; Rosenquist, 1966 ; Schoenwolf & Sheard, 1990 ; Bortier & Vakaet, 1992 ; Garcia-Martinez et al., 1993). When cranial hypoblast from pre-streak stages (EK XII/XIII) or from mid-streak stages (HH3) or the definitive endoderm from late streak stages (HH4) was grafted on whole blastoderms, neither morphological alteration nor ectopic expression of GANF was elicited. Labelling with DiI demonstrated that the hypoblast cells remained together at the position of grafting during the incubation (which we have also seen in the present study). Transplants of Hensen's node (HH3+/HH4) on whole blastoderms led to the induction of a neuroectodermal structure with a strong expression of GANF in its cranial margin. Grafting of the young head process (HH4+) to the lateral cranial area pellucida caused a thickening of the epiblast and an induction of GANF expression in juxtaposed cells. Formerly, it was generally believed that the neural plate is only formed in the upper layer by an inductive interaction with the underlying chordamesoderm. However, animal caps isolated from amphibian embryos, injected with a dominant negative activin receptor, spontaneously express neural markers such as the neural cell adhesion molecule N-CAM without any signal from the mesoderm (Lemaire, 1992). More recently *in situ* hybridisation studies demonstrated that Otx2 function is required in the murine primitive visceral endoderm for the induction of forebrain and midbrain (Rhinn et al., 1998). A secreted molecule named *Cerberus*, which is expressed in anterior endoderm, has the property to induce ectopic head structures when micro-injected into ventral regions of *Xenopus* embryos (Bouwmeester et al., 1996 ; Bouwmeester, 1997). The patterning of the chick forebrain *Anlage* by the prechordal plate has been described by Pera & Kessel (1997). According to these authors also, the avian neural plate is evident before the first mesendodermal or axial mesodermal cells ingress, excluding the prechordal plate and the notochord as primary sources for neural induction. A previous study indicated that avian endophyll (from unincubated blastoderms) can induce a (pre)neural plate, with or without neural folds in the upper layer of the caudal marginal zone, where normally no endophyll is present (Callebaut et al., 1999a). By interaction with sickle endoblast arising from Rauber's sickle (the early gastrulation organizer : Callebaut & Van Nueten, 1994 ; Callebaut et al., 1997a) or from Hensen's node (a later avian organizer : Waddington, 1932), endophyll orients or re-orients the head region and the caudocranial direction of an induced miniature embryo (Callebaut et al., 1999a). It was proposed by Perea-Gomez et al. (2001) that the anterior visceral endoderm in the mouse embryo protects anterior embryonic regions from signals that promote

posterior development. There seems to be much homology between the visceral endoderm in the mouse and the avian sickle endoblast. Both deep layer structures move actively before and during gastrulation and are progressively replaced by definitive endoderm during gastrulation (in the mouse : Lawson & Pedersen, 1987 ; in the chicken : Callebaut & Van Nueten, 1994 ; Callebaut et al., 1997b). The cranial part of the late avian streak is capable of direct neural induction, and its tip, Hensen's node can induce an anterior neural identity. This latter activity leaves the node together with the cells representing the anterior mesendoderm (Boettger et al., 2001). During early gastrulation, cells invaginate through the tip of the growing streak and spread radially to form the definitive (gut) endoderm (Vakaet, 1970). During this radial expansion, the latter definitive endoderm pushes the sickle endoblast also radially (Callebaut & Van Nueten, 1994) (Figs 6, 10D). The cranial hemi-circular sickle endoblast slides under upper layer cells that will transform into the also hemicircular neural plate *Anlage* (Bortier & Vakaet, 1992). The latter cells are localized close to the former anti-sickle region exactly in the concavity of the cranially-displaced endophyllic crescent (Figs 6, 10E). The remaining more caudal sickle endoblast is localized under the upper layer, which will give rise to the primitive streak-forming area, localized in the area centralis region. This different evolution in the cranial (anti-sickle) region versus the central (area centralis) region can probably been explained by the different reactivity in these two upper layer regions observed in the present study. We cannot exclude the possibility that some endophyll is also included in the transplanted sickle endoblast but surely it contains no definitive endoderm since the sickle endoblast was excised before the definitive endoderm appears. That no inducing effect was observed by apposition of quail sickle endoblast on the anti-sickle region of whole unincubated chicken blastoderms, can probably be explained by the domination and inhibition at long distance (positional information) by the still present autochthonous Rauber's sickle or junctional endoblast (Callebaut et al., 2000c). Indeed in the present study we observed that if a fragment of quail sickle endoblast was placed on the anti-sickle region of an unincubated chicken blastoderm from which the Rauber's sickle was totally or subtotally removed, then often starting from this anti-sickle region an embryo was induced presenting both gastrulation and neurulation phenomena but no blood island formation. Rauber's sickle can thus quantitatively dominate or inhibit the ectopically-placed hierarchically-submitted sickle endoblast (belonging to the same cell lineage). Our study also indicates that via its outgrowth (sickle endoblast), Rauber's sickle also influences at distance the formation of the neural plate. The absence of neural induction after the grafting experiments with hypoblast on whole blastoderms by Galera & Nicolet (1969) and by Knoetgen et al. (1999a, b) can probably also be explained by the full presence of Rauber's sickle material. This indicates also that the earlier conclusions from grafting experiments on whole unincubated blastoderms (containing Rauber's sickle, a primary major organizer) or on primitive streak blastoderms (containing Hensen's node, a secondary major organizer) must be reconsidered. Thus, to study correctly interactions between different combined parts of the avian blastoderm, it is often necessary to culture them in isolation from other parts or cell groups of the blastoderm. Therefore we cannot agree with either of the conclusions of Knoetgen et al (1999b) that the endoblast on its own elicits any detectable change in the adjacent host ectoblast after transplantation, or that the avian organizer is confined to Hensen's node only.

When an anti-sickle region was cultured in isolation no differentiation was seen (Callebaut & Van Nueten, 1995). After placing an endophyll fragment on the naïve upper layer of isolated anti-sickle regions (Callebaut & Van Nueten, 1995), we observed the induction of a (pre)neural plate (often with a median neural groove and lateral neural walls). These experiments incited Foley et al. (2000) to study the eventual role of the early deep layer (endophyll and/or sickle endoblast) on the expression of the molecular markers Sox3 (Uwanogho et al., 1995) and Otx2 (Bally-Cuif et al., 1995) in the upper layer. From stage 6-7 HH on, Sox3 is specifically expressed in the entire chicken neural plate and Otx2 is expressed throughout the forebrain and midbrain. Foley et al. (2000) found that the early deep layer regulates an early transient phase of Otx2 and Sox3 expression in the adjacent upper layer. Therefore they concluded that the early deep layer does not induce neural tissue or forebrain definitively. However, their transplantation experiments were not performed on Rauber's sickle- or junctional endoblast-free blastoderm fragments but on whole blastoderms. As seen in earlier studies and in the present study, both structures have dominating and suppressive potencies (Callebaut et al., 2000c). Recently Knezevic & Mackem (2001) found evidence that two genes, later associated with the gastrula organizer (Gnot-1 and Gnot-2), are induced by the deep layer signals in prestreak embryos. According to the latter authors, these genes could perhaps regulate axis formation in the early embryo, which could also explain the induction of a streak in the isolated central part of the area centralis by sickle endoblast (Callebaut et al., 2003b study). In our fate map of Anlage fields in unincubated chicken blastoderms (Callebaut et al., 1996a), we have shown that the *Anlage* of the future central nervous system extends as a large shield-like surface structure from just caudally from the centre of the area centralis to a short distance from the middle of the concavity of Rauber's sickle (Fig. 13A). This neural plate *Anlage* is thus exactly localized over the endophyll region. During early gastrulation, the cranially-growing sickle endoblast pushes and penetrates the endophyll (Callebaut et al., 1997b). At the same time, the primitive streak-forming upper layer area displaces this predisposed neural plate *Anlage* in a cranial direction (Callebaut & Van Nueten, 1996). So the prediposed neural plate *Anlage* and the associated endophyll (between both exist narrow cellular bridges : Callebaut et al., 1999b) move side by side simultaneously (the so called "mouvements simultanés" reported by Pasteels, 1937). So, both structures remain in prolonged contact during gastrulation. This can probably explain why finally the influenced upper layer, (successively by endophyll and sickle endoblast) during further normal development (after stabilization of its preneural stage by prechordal mesendoderm and cranial head process) will definitively form a central nervous system cranially to the Hensen's node where sickle endoblast is localized in the concavity of the endophyllic crescent (Bortier & Vakaet, 1992) (Fig. 13B) and at distance from the junctional endoblast (Fig. 6). It is indeed remarkable that the shield-like predisposed Anlage field of the whole central

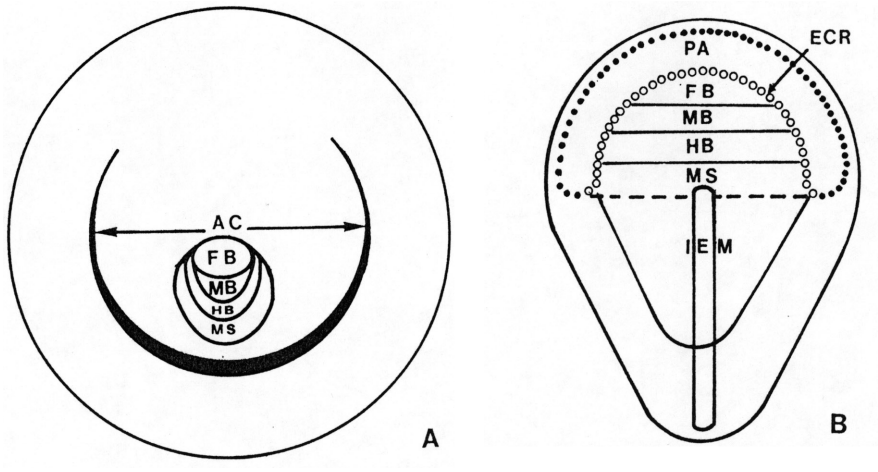


Fig. 13. - Comparison of the disposition of the neural plate Anlage (and its subdivisions), on dorsal views in the upper layer of A. the unincubated chicken blastoderm (according to CALLEBAUT et al., 1996a) and of B. the stage 4-5 (HAMBURGER & HAMILTON) chicken blastoderm (according to BORTIER & VAKAET, 1992) ; FB : forebrain Anlage ; MB : midbrain Anlage ; HB : hindbrain Anlage ; MS : Anlage of the medulla spinalis ; AC : area centralis enclosed by Rauber's sickle ; seen in transparency is the proamnion (PA), a broad sickle-shaped cavity cranially from the endophyllic crescent (ECR) ; IEM : intraembryonic mesoblast Anlage in the upper layer.

nervous system is first localized in the upper layer of the caudal hemicircular half of the blastoderm at the unincubated stage (CALLEBAUT et al., 1996a), whilst after approximately one day of culture (Stage 5-6 of HAMBURGER & HAMILTON) the same upper layer part (but now definitively determined as neural tissue) is localized in the cranial hemicircular half of the blastoderm (cranially from Hensen's node : BORTIER & VAKAET, 1992) (Fig. 13B). A previous study (CALLEBAUT et al., 2003b) and the present one suggest the existence of a temporo-spatially bound cascade of gastrulation and neurulation phenomena and blood island formation in the avian blastoderm, starting from Rauber's sickle, the primary major organizer. Successively, we observed the following steps : 1) induction of a primitive streak in the upper layer by signalling molecules, secreted by Rauber's sickle or by sickle endoblast diffusing over a long distance in the blastoderm eventually through an interposed vitelline membrane (CALLEBAUT et al., 2003a), 2) differentiation of junctional endoblast from Rauber's sickle by interaction with the upper layer and/or endophyll since the interposition of a vitelline membrane inhibits this ; this junctional endoblast is indispensable for blood island formation (CALLEBAUT et al., 2002) ; 3) differentiation of sickle endoblast moving cranially from Rauber's sickle into/and with the endophyll (CALLEBAUT et al., 1997b, 1999a). In the upper layer of the blastoderm, sickle endoblast induces proximally (i.e. close to Rauber's sickle) the formation of a primitive streak, whilst distally (i.e. diametrically opposite to Rauber's sickle) it induces a (pre)neural plate. The ingrowth of sickle endoblast into the endophyll (CALLEBAUT et al., 1999a) seems to be the histological basis by which the caudocephalic orientation of the neural tube and head region is directed towards the endophyll. So, Rauber's sickle material by its cell lineage (sickle endoblast) also influences neurulation at distance (in space and time). Hensen's node must be considered as a secondary major organizer, which becomes functional only after Rauber's sickle.

Already in 1913, SCHOUTTE proposed that the spacing of leaves in plants was the result of mutual inhibition of leaf primordia, such that each new leaf can only appear at a certain distance from the preceding one. Also WIGGLESWORTH (1940) interpreted the spacing of bristles on an insect and the insertion of new bristles in the largest interstices in this way. To account for the long range effect of small specialized regions and for the spatial continuity observed after many experimental interferences in early sea urchin development, hydroid regeneration and development of the chick limb, the "positional information" scheme was proposed by WOLPERT (1969, 1981). As during the spacing of leaves in plants, blastodermal cell groups of the same cell lineage (Rauber's sickle, junctional endoblast and sickle endoblast) have inhibiting effects on their like. So, usually in the limited surface of one and the same blastoderm (3 mm diameter), only one of them may retain its organizing capacities. That after (sub)total removal of the autochthonous Rauber's sickle still an embryo can develop, can be explained by different reasons : 1) the removal of the Rauber's sickle material was incomplete, so from the remnant of a sickle horn often a transversely oriented streak develops ; indeed the extent of the circumference of a complete Rauber's sickle is often considerably underestimated. We have shown that it is not only found in the caudal quadrant of the germ, but also fragmentarily in the whole periphery of the lateral quadrants (CALLEBAUT et al., 2000b) (Fig. 13A). Thus in cases of incomplete removal of Rauber's sickle and after further culture, junctional endoblast accompanied by blood islands will appear in the involved area ; 2) some sickle endoblast derived from the autochthonous Rauber's sickle has already migrated into the caudal part of the area centralis ; 3) signalling molecules secreted by the autochthonous Rauber's sickle before its removal have already diffused sufficiently far enough into the caudal part of the area centralis to start streak formation. Moreover the presence of endophyll in the area centralis can explain why also a neural plate can develop.

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