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Review

Relationship and interaction between the early avian embryo and neighboring egg structures: Renewed insights

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ABSTRACT

Yolk and ooplasmic labeling permitted us to bridge the gap between the radially symmetrical ooplasmic structures (α, β, γ, δ-ooplasms) and their fate during early embryonic development. At the moment of bilateral symmetrization (characterized, long before the formation of the primitive streak, by the appearance of Rauber's – Koller's sickle), an oblique sickle-shaped uptake of γ-ooplasm (Rauber's sickle material, containing Vg1) and δ-ooplasm (endophyll) into the deeper part of the avian blastoderm occurs. Contrary to common belief, the Nucleus of Pander (superficial part) plays an important role during early development (before laying) by giving rise to the deep δ-ooplasm-containing endophyllic layer (inducing preneurulation and directing gastrulation) and from which the primordial germ cells will segregate into the germinal crescent. Rauber's sickle (containing Vg1) and not the caudal marginal zone is the early primary organizer of the avian blastoderm. It induces the Anlage fields in the upper layer cells localized within its concavity (intramural effect). Later, after formation of a primitive streak, Rauber's sickle induces the formation of the coelomo-cardio-vascular system at its outer side in the caudal marginal zone (extramural effect). We found evidence that avian conjoined (Siamese) twins are formed by fusion (parapagus) and not by fission. The autochthonous Rauber's sickle has an inhibitory effect (competitive inhibition) on exochthonous Rauber's sickle material or sickle endoblast placed on the anti-sickle region of whole blastoderms. The spatial spreading of Rauber's sickle material in relationship with the neighboring upper layer influences left/right asymmetry and determines regulation *in ovo* versus mosaic (hemi-primitive streak) development *in vitro*. The real significance of the caudal marginal zone versus Rauber's sickle is discussed. The penetration of radioactively labeled egg white-derived material through the vitelline membrane and its massive selective accumulation into the epigerminal, the subgerminal space and perigerminal yolk region, is demonstrated.

KEYWORDS: avian embryo, ooplasmic determinants, Rauber's sickle, Nucleus of Pander, albumen

INTRODUCTION

At the end of its final oocytal growth period (postlampbrush stage) [1], the ooplasm of the avian germ disc exhibits both histochemically and autoradiographically a concentric, radially symmetric distribution. DNA- and RNA-containing material is bound to mitochondria forming the so-called ticos (tritiated thymidine-incorporating cytoplasmic organelles) [2, 3, 4] (Fig. 1). After fertilization the early avian germ is localized in the so-called cicatricular region which is visible alive (Fig. 11), from the surface of the egg yolk as a circular whitish disc below which a central denser white

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yolky structure (called Nucleus of Pander) [5] is visible. In this cicatricular region two important egg substances play, after incubation, a role during early embryonic development:

1) Below the vitelline membrane different ooplasmic layers and accompanying yolk components are found [1, 6]: α, β, γ and δ-ooplasms with an onion peel distribution (Fig. 2), built up during late oogenesis.

2) Surrounding and exterior to this vitelline membrane, the transparent egg white (albumen) is discerned.

In the also very yolk-rich Selachian eggs, Wintrebert [7] demonstrated that the caudo-cephalic axis of the future embryo was not preformed but determined at random by a temporary oblique positioning of the germ disc on the surface of the yolk ball, suggesting mechanical interaction between germ and yolk. The influence of the "yolk factor" ("mur vitellin") during the earliest development of the avian embryo was already described in the work of Dalcq and Pasteels [8].

Callebaut [9, 10] described the oblique formation of the subgerminal space in quail eggs by the combined action of the earthly gravity and the positioning of the egg yolk. This has a decisive inductory influence on the further development of the blastoderm as the result of the unequal gradual caudo-cephalic distribution of the deep blastoderm components (Rauber's sickle and endophyll) (Fig. 2) This incited Eyal-Giladi [11] to describe, by a comparative study, the effect of the quantity of yolk elements on the formation of the axis in chordates. Arendt and Nübler-Jung [12] made a detailed comparative morphology study of the evolution of gastrulation in yolk-rich amniote eggs. So they were able to rearrange gastrulation in the name of yolk. We describe here the composition and function of the different morphological structures of the young avian blastoderm derived from the original ooplasmic components (α, β, γ and δ) formed earlier during oogenesis.

New [13] has found that avian blastoderms grown *in vitro* on pieces of vitelline membrane, supported over albumen, remove fluid from the albumen and secrete it from their endodermal surfaces. This seems to be a mechanism of formation of sub-blastodermic fluid.

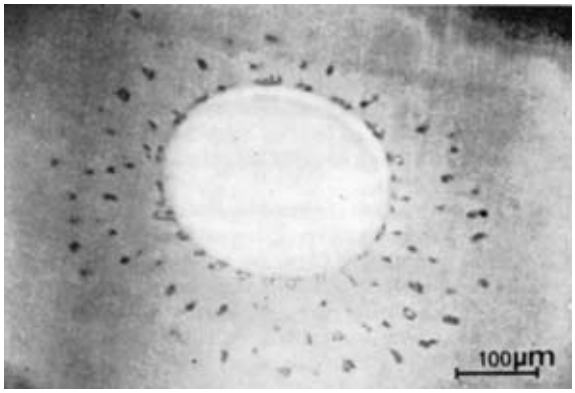


Fig. 1. Tangential section through the germ disc of a quail oocyte during the postlamp-brush stage after Toluidine blue staining; the RNA rich, mitochondrial DNA containing subcortical ooplasmic organelles (ticos) are localized in concentric circles but also in radially placed rows. Bar = 100 µm.

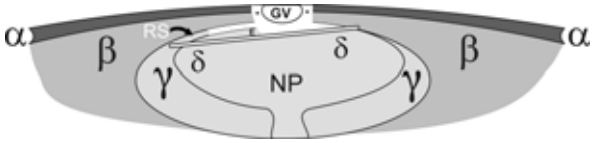


Fig. 2. Schematic drawing of a midsagittal section through a quail oocyte just before maturation, representing the four ooplasmic regions with an onion-peel organization: the α-ooplasm, most superficial and peripheral; the deeper β-ooplasm; the γ-ooplasm surrounding at distance the Nucleus of Pander; and the deepest central δ-ooplasm in the Nucleus of Pander. The oblique split represents the spatially unequal formation of the subgerminal space, resulting in the spatial oblique uptake in the deeper part of the blastoderm of peripheral γ-ooplasm forming the caudal Rauber's sickle (RS), and the uptake of the δ-ooplasm, forming endophyll (E) in the future centro-caudal region of the blastoderm; GV, germinal vesicle surrounded by a circle of ticos in a region where the 4 ooplasms converge (unstained perinuclear zone).

We describe here other mechanisms of transport of fluid (derived from the albumen) through the vitelline membrane by the use of radioactively labeled precursors.

In general, in the present study, we investigated the physico-chemical interaction between at one

side the ooplasm with yolk and at the other side the egg white with the young avian embryo.

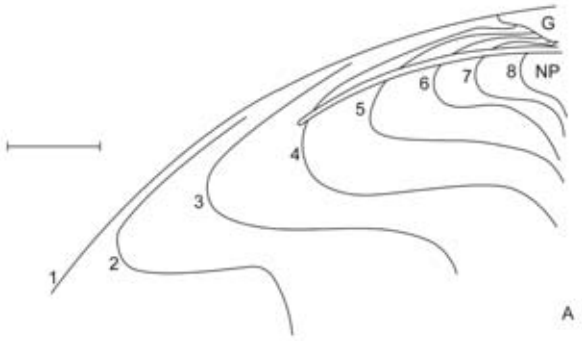
Ooplasm and yolk

Radioactive or trypan blue induced oocytal labeling of the yolk and/or ooplasmic layers were performed by injecting the mother quail at different days before laying. After fixation *in toto* of the avian egg yolk ball (with germ disc still *in situ*) we could demonstrate continuity between the broad macroscopic yolk layers (exterior to the germ disc) and the narrow microscopic ooplasmic layers in the germ disc [1]. Thus the germ disc do not floats separately on the top of the yolk ball but forms an integral part of it.

By such successive labellings of the layers it was possible to elucidate some important structural relationships in the avian germ. In the oocytal germ disc, at the end of the final growth period, the successively labeled layers have the general shape of onion peels in/or around the Nucleus of Pander (Fig. 2). In the peripheral superficial part we find the very mobile α ooplasm which forms cleavage furrows, so separating the first blastomeres. After the cleavage period this α ooplasm disappears and only the three deeper and more central ooplasms remain, forming the three elementary tissues in the laid unincubated blastoderm (Figs. 3A, 3B). The somewhat more central β ooplasm settles mainly in the upper layer and will form the stem cells of the embryo proper after segregation from the endophyll [14].

As the result of the oblique formation of the subgerminal space [9, 10] a three-axial germ appears (dorsoventral, caudo-cephalic, latero-lateral or left-right).

At the same time in the area pellucida a caudocephalic tissue gradient becomes visible, constituted by the two deeper layers (the sickle shaped γ ooplasm, forming Rauber's sickle and the centro-caudal δ-ooplasm, forming endophyll) (Fig. 3C).



Figs. 3A, 3B. Comparison of the autoradiographic localization and aspect of radioactively labeled yolk layers in a full grown quail oocyte (A) with the similar labeling in the germ disc of an unincubated quail egg developed after fertilization of the latter (B).

Fig. 3A. Combined scale drawing showing the approximate level of the future subgerminal cavity (represented by a curved split) at the moment of laying, in the animal pole of a quail oocyte just before maturation. The successively labeled yolk layers (after daily maternal injection of ^{3}H-tyrosine), represented by lines 1 to 8 (1 to 8 days after injection) are spherically disposed and show no eccentricity. G: germinal vesicle; NP: Nucleus of Pander; scale line: 200 µm.

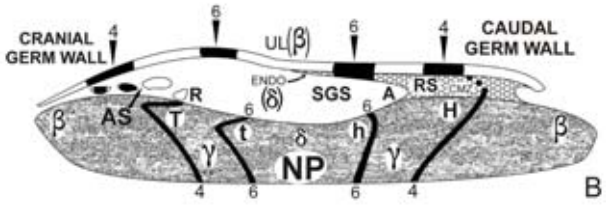


Fig. 3B. Schematic representation of a mediosagittal section through an unincubated quail germ disc on its egg yolk presenting only two labeled ooplasmic layers, corresponding with layers 4 and 6 in Fig. 3A. The two labeled layers show now permanent eccentricity under influence of a combined action of gravity and egg rotation *in utero*; SGS: subgerminal space; AS: anti-sickle formed of loose yolk masses and cells in the cranial recessus (R) of the subgerminal space; A: axilla; armpit-like caudal part of the subgerminal space; END: arrow indicates endophyll (δ-ooplasm); RS: Rauber's sickle; CMZ: deep part of caudal marginal zone; UL: upper layer; in the central part of the subgerminal ooplasm we see the Nucleus of Pander (NP) surrounded by labeled ooplasmic layer 6 and at distance by labeled layer 4: note the caudocranially oriented eccentricity, forming cranially a toe-shaped structure (T or t) and caudally a heel-like structure (H or h); layer 4 ends caudally in the caudal marginal zone whilst cranially layer 4 is interrupted in the cranial recessus and in the antisickle region: this demonstrates a caudocranially oriented eccentricity in the unincubated blastoderm and eccentric formation of the subgerminal space.

They form the so-called extraembryonic tissues which by induction will influence the differentiation of the neighboring upper layer cells (stem cells) during the development of the embryo proper. The latter three elementary tissues are thus early components of the unincubated avian blastoderm (Figs. 3B, 3C) and are different from the three later-formed classical germ layers (epiderm, mesoderm, gut endoderm), originally described by Pander [5] or Von Baer [15]. These classical three germ layers are derived from the original upper layer.

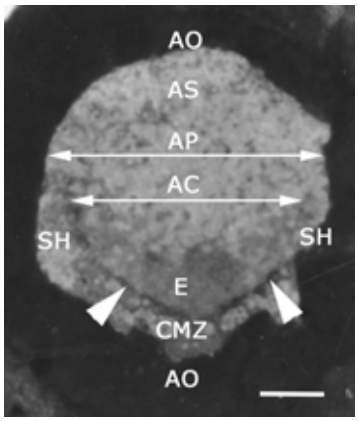


Fig. 3C. Transparent area pellucida (AP) from a living chicken blastoderm, seen from below, showing deep layer components, adherent to the deep side of the transparent upper layer. E: endophyll in the caudal concavity of the denser Rauber's sickle (indicated by white arrowheads); SH: sickle horns; AC: area centralis in the concavity of Rauber's sickle; AS: anti-sickle; AO: area opaca. The caudal marginal zone (CMZ) is part of the upper layer just peripheral to Rauber's sickle, in which the first blood islands will develop after incubation; under influence of Rauber's sickle. Bar = 1 mm.

Rauber's sickle

The avian unincubated blastoderm was usually considered to be completely separated from the underlying yolk ball [16]. However we demonstrated that the yolk labeling (γ-ooplasm) in the Rauber's sickle is continuous with the yolk labeling in the underlying ooplasm (Fig. 4) [6]. Rauber's sickle forms thus a kind of caudal "socle" by which the blastoderm is fixed to the underlying ooplasm and yolk (Figs. 3B, 4A, 4B). It also must be considered as the most vegetal part of the blastoderm (homologous to the Nieuwkoop centre in amphibians) since large "islands" of underlying ooplasm are engulfed by voluminous phagocytotic cell protrusions of the Rauber's sickle cells [6, 10]. Also by this process, during early incubation, Rauber's sickle becomes progressively more massive by uptake of underlying ooplasm. Later it forms the V-shaped junctional endoblast, usually visible alive from the surface in the caudal region of the blastoderm. In contrast, the cranial part of the blastoderm (the anti-sickle region) contains no Rauber's sickle material and forms an uncommitted narrow upper layer, lying loosely and freely over the underlying yolk [9, 10]. Rauber's sickle and not the caudal marginal zone cells induce the formation of the definitive endoderm and mesoblast, via the primitive streak [17]. We could not confirm the conclusion of Eyal-Giladi *et al*. [18, 19] that caudal marginal zone cells migrate into the area centralis via Rauber's sickle. Indeed in our experiments neither the Rauber's sickle (or junctional endoblast) nor the area centralis were seen to be labeled after grafting in the caudal marginal zone. Since Eyal-Giladi and coworkers used vital dyes for their experiments, we think that this stain did not remain localized *in situ* at the application site and leaked into the surrounding tissues. More over in the experiments of Eyal-Giladi and colleagues the distinction between caudal marginal zone and Rauber's sickle was not always separately visible and often both structures were transplanted together. The Rauber's sickle is a very important limiting structure between the area centralis [20, 16] and the caudal marginal zone. By excision at different sites of small fragments from unincubated chicken blastoderms and replacement by isotopic fragments from unincubated quail blastoderms (functioning as biological markers) [21] and culture we made the first mosaic-like complete map of the Anlage fields in the unincubated avian blastoderm.

All these sickle-shaped Anlage fields are found in the upper layer of the caudal half of the area centralis [17]. In the upper layer of the area marginalis peripheral to the Rauber's sickle, by this way no gastrulation nor neurulation phenomena could be obtained. So we could demonstrate that the Anlage field of the definitive gut endoderm (which is derived from the upper

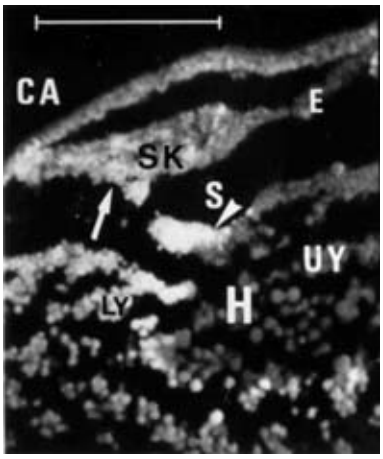


Fig. 4A. Fluorescence micrograph of a mediosagittal section through the future caudal part (CA) of the quail germ (obliquely oriented during approximately 14 h), developing in the fourth labeled prelaid egg after a maternal injection of trypan blue; S: subgerminal space separating the germ (above) from the uncleaved ooplasm (below); LY: trypan blue labeled yolk layer in uncleaved ooplasm with in its prolongation, indicated by an arrow, the labeled sickle of Rauber (SK) in the blastoderm. H: caudal heel-like part of unlabeled uncleaved ooplasm (UY) surrounded by a short hook (arrow head) of labeled ooplasm. At the level of the arrow the tissue is artificially disrupted during fixation; E: unlabelled endophyll in continuity with the labeled sickle of Rauber. Scale-line = 200 µm.

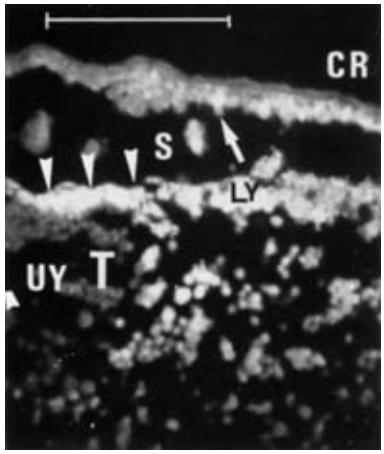


Fig. 4B. Cranial part (CR) of the same section as Fig. 4A. The quantity of ooplasm taken up by the germ is less voluminous then in the caudal part, and more labeled uncleaved ooplasm (3 arrow heads) remained localized below the widely open subgerminal space (S); T: cranial toe-like part of unlabeled uncleaved ooplasm (UY) surrounded by a much longer hook of labeled ooplasm (LY). No sickle of Rauber-like structure has developed; same magnification as Fig. 4A.

layer) [22, 20] is localized in the most caudal upper layer part of the area centralis, just centrally to the Rauber's sickle (Fig. 5). We found evidence that initially a kind of ooplasm-bound preformation (or predisposition), disposed in concentric circular ooplasmic layers, exists both in the full grown oocyte and in the early germ. Under influence of mechanical and gravitational forces an early excentricity is created with unequal oblique uptake and segregation of ooplasmic determinants in the germ [23]. This excentricity has been confirmed by the use of molecular biology markers. Not only the fate but also gene expression patterns indicate a role of Rauber's sickle as an early avian organizer [24]. Accordingly cells near Rauber's sickle and in its concavity express a whole battery of genes related to genes associated with the amphibian organizer, such as Gsx, Gsc, c Not 1, c Not 2, Hnf 3 beta, otx 2 and chordin [25, 26, 27, 28, 29, 30]. All these

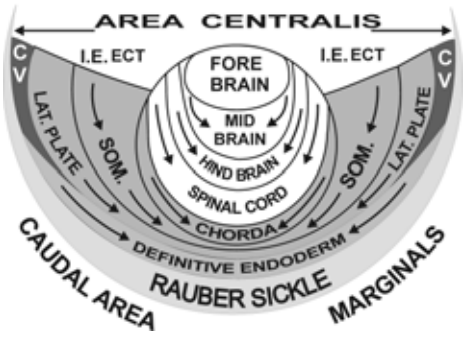


Fig. 5. Schematic representation of the mosaic-like localization of the predisposed (not definitively committed) anlage fields (in good order but with possible partial overlapping of neighboring parts) in the upper layer of the area centralis of a chicken (*Gallus domesticus*) unincubated blastoderm. Note the general eccentric sickle-shaped aspect of the anlage fields in the area centralis. There is an obvious parallelism between the sickle shape of the anlage fields in the upper layer (UL) and the ovoid central subgerminal ooplasmic layers. The curved arrows on the anlage fields indicate the logically previsible converging movements of the upper layer during the ensuing gastrulation and neurulation. CV, anlage of the coelomo-cardiovascular system localized between definitive endoderm and lateral plate anlagen.

expression domains are initially sickle-shaped (parallel with Rauber's sickle) i.e. spread more or less broadly and transversely to the future longitudinal axis (primitive streak) These expression domains are localized in the concavity of Rauber's sickle, where we have localized the similarly sickle-shaped Anlage fields in the upper layer of the unincubated chicken blastoderm [17].

We demonstrated the fundamental influence of the spatial distribution of Rauber's sickle material with reference to the upper layer cells on major developmental processes as regulation (*in ovo*) or mosaicism (*in vitro*) (Fig. 6) in avian unincubated blastoderms [31, 32].

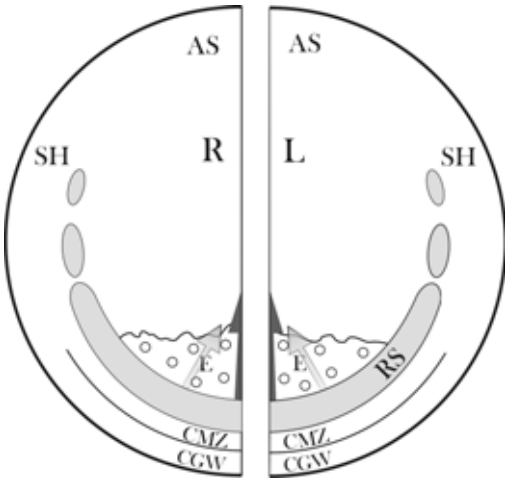


Fig. 6. Demonstration *in vitro* of mosaicism in birds: schematic representation of a symmetrically hemi-sectioned (with reference to the Rauber's sickle) unincubated blastoderm, to indicate its distinct components; AS, anti-sickle region; RS, half Rauber's sickle; SH, sickle horn tops. Although containing Rauber's sickle material, the sickle horn tops behave differently from the median part of Rauber's sickle (Callebaut *et al.* 2010b). R, right hemi-blastoderm; L, left hemi-blastoderm; E, endophyll. The thick, half-arrows represent the formation, after culture, of hemi-primitive streaks at the cut edges of the blastoderm half. CMZ, caudal marginal zone; CGW, caudal germ wall (area opaca). The transparent arrows indicate the hypothetical localization of bilateral symmetrical primitive streaks with reference to the half Rauber's sickles, if only regulation phenomena would occur (i.e. according to the median line, going through the three elementary tissues in one half blastoderm).

Moreover the asymmetric spreading of Rauber's sickle material influences left/right asymmetry [34] (Figs. 8A, 8B).

The sickle shaped distribution of the γ ooplasm-containing cells (RS material) thus not only Rauber's sickle, is by positional information the early primary organizer of the primitive streak (intramuros: in the concavity of Rauber's sickle) [33] but afterwards also the development of the coelomo-cardiovascular system (extramuros) is organized by Rauber's sickle material (Fig. 7). determines the plane of bilateral symmetry but also it seems to play a role in the establishment of the left-right asymmetry (Figs. 8A, 8B). That Vg1 (the axis inducer) is present in the avian Rauber's sickle (inclusive/the sickle horns) and not in the caudal marginal zone has recently been demonstrated by Bertochini and Stern [35].

Sickle endoblast

Sickle endoblast forms an unicellular far-extending layer proliferating from the medial rim of the Rauber's sickle or junctional endoblast in a centripetal and cranial direction during early

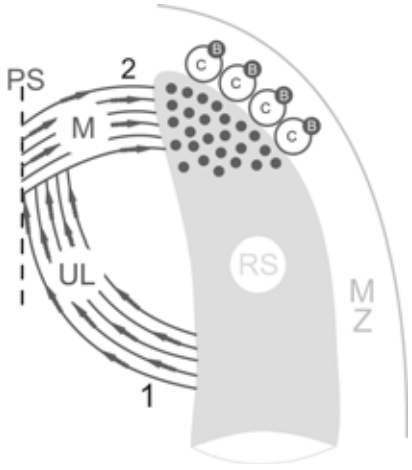


Fig. 7. Schematic representation (ventral view) of the normal formation of the coelomo-cardiovascular system in the chick embryo after 2 successive inductions by Rauber's sickle. (1) Upper layer cells localized in the concavity of the RS form, after ingression through the primitive streak (PS), a mesoblast mantle (M) that slides peripherally (2) between the RS and the epiblast into the marginal zone (MZ) to form coelomic vesicles (C) and associated blood islands (B).

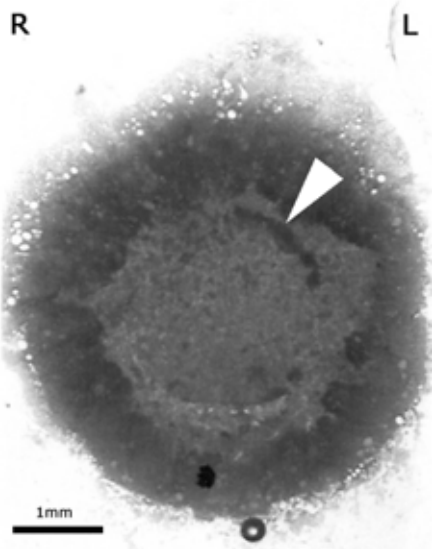


Fig. 8A. Ventral view of unincubated chicken blastoderm from which the middle part of Rauber's sickle is displaced in the left cranial quadrant in the prolongation of the sickle horn giving an asymmetric distribution of Rauber's sickle material.

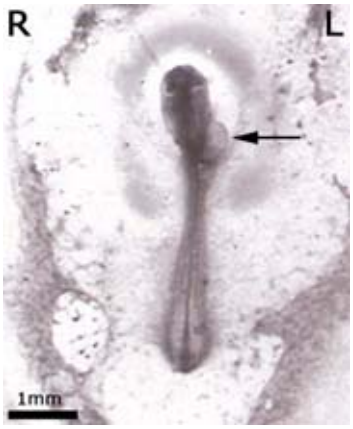


Fig. 8B. Ventral view after 41 h of culture: the embryo presents *situs inversus cordis* with ventricular looping directed to the left (arrow).

incubation. [36, 37] (Fig. 9). Principally it contains γ-ooplasm since it is derived from Rauber's sickle material. During early incubation the sickle endoblast pushes the endophyll in a cranial direction which so forms the endophylic crescent (Fig. 9). Both endophyll and sickle endoblast have respectively an influence on neurulation and gastrulation (Fig. 10). When quail

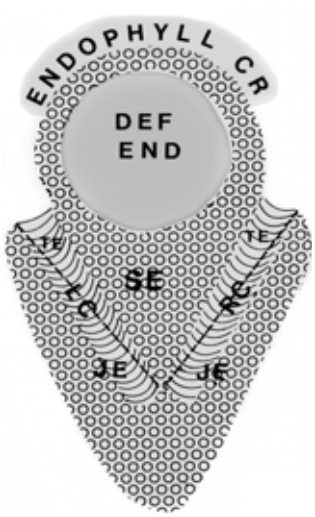


Fig. 9. Schematic drawing of the anchor-shaped spreading seen from the dorsal side after removal of the upper layer and primitive streak region, at stage 3-4, of the two cell lineages (SE: sickle endoblast; JE: junctional endoblast), derived from Rauber's sickle; the small circles represent cellular 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 in which primordial germ cells first appear.

sickle endoblast is placed on the isolated anti-sickle region of an unincubated chicken blastoderm in culture, an early neural plate develops. By contrast, when a piece of quail sickle endoblast is placed on the anti-sickle region of a whole unincubated chicken blastoderm in culture it has no inducing effect [38]. This observation explains the contradictory conclusions obtained by earlier authors, that have studied the effect of the deep layer on the upper layer [39, 40, 41]. This indicates that Rauber's sickle dominates or inhibits ectopically-placed sickle endoblast which is derived from the same cell lineage (γ-ooplasm containing). This sickle endoblast, if withdrawn from the influence of Rauber's sickle, has gastrulation and/or neurulation inducing potencies on the upper layer of the unincubated blastoderm, but it has no influence on blood island formation. The homeobox gene cHex is expressed in Rauber's sickle and sickle endoblast [42].

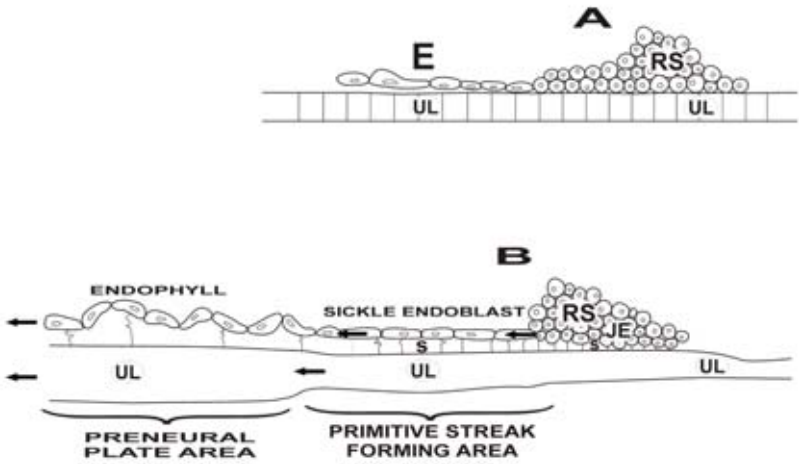


Fig. 10. Schematic drawing of the neurogastrulating effect on the upper layer (UL) *in vitro* after placing endophyll (E) in the immediate neighbourhood of Rauber's sickle (RS) material. At the start of the culture, these 3 elementary tissues present in the unincubated avian blastoderm are in close contact. After *in vitro* culture the endophyll on its own influences the differentiation of the UL: the endophyll induces the preneural-plate-thickening in the neighboring part of the UL, whilst a sheet of sickle endoblast grows from Rauber's sickle in its direction and pushes it away from RS: the preneural plate moves simultaneously and side by side with the endophyll in the same direction. At the same time UL material coming from laterally converges and accumulates in the midline in front of RS and constitutes the primitive streak forming area; note the spaces (S), separated by extensions, between UL and sickle- or junctional endoblast (JE).

cHex transcripts were also detected within blood islands beginning at stage 4 (H and H, 1951) and in extraembryonic and intraembryonic vascular endothelial cells. Because 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 cHex gene.

Influence of sickle endoblast on neurulation and gastrulation

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 [43]. 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.*, [40, 41] 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 [44, 45, 46, 47, 48]. The patterning of the chick forebrain Anlage by the prechordal plate has been described by Pera & Kessel [49]. 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. During early gastrulation, cells invaginate through the tip of the growing streak and spread radially to form the definitive (gut) endoderm [16]. During this radial expansion, the latter definitive endoderm pushes the sickle endoblast also radially [36] (Fig. 9).

Nucleus of Pander

The Nucleus of Pander (localized in the centre of the central subgerminal ooplasm) contains primordial yolk globules i.e. the earliest peripherally-assembled avian yolk [1, 50]. It forms a dense cushion below and in the depth of the blastoderm (Fig. 11).

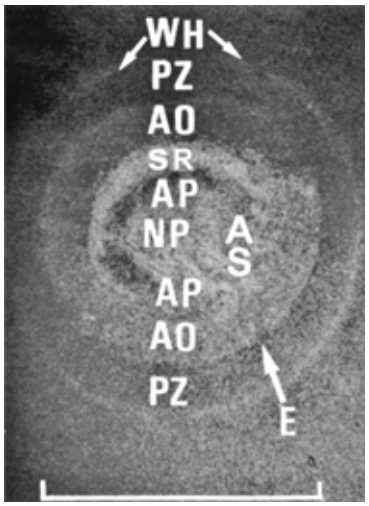


Fig. 11. Surface view of a living unincubated quail germ *in situ* on top of its egg yolk ball. AO, area opaca with clear-cut edge (E); AS, anti-sickle region containing numerous disrupted deep ooplasmic masses and cells; NP, remaining deeper part of the Nucleus of Pander, visible through the transparent area pellucida (AP); PZ, perigerminal clear zone limited by a white halo (WH), forming the paragerminal β tubulin-containing ooplasm; SR, sickle of Rauber. Bar = 3 mm.

It was a subject of debate for physiologists during many centuries and many supporters of preformation saw it as the real germ of the fetus [51]. Malpighi [52], adhering to a preformistic ovism, held the idea that the embryo, initially localized in the Nucleus, emerged from it in the course of development. According to Wolff [53] there was no embryo at all in the Nucleus. It was Pander [5] who realized for the first time that the real embryo is formed by the thin membrane (blastoderm) over it. In that time it was unimaginable that a membrane could give rise, as touched by a magician, to a whole embryo. This observation was in favor of the epigenetic (transformation) theory. So the Nucleus of Pander, until very recently, was considered as a plug of whitish yolk, with no particular significance for development and whose function is purely nutritive. However we have shown by labeling of the concerned deep ooplasmic layers that the superficial part of the Nucleus of Pander is taken up in the germ, forming the δ-ooplasm-containing endophyll from which primordial germ cells will arise during the early somite stage [54, 55]. The Nucleus of Pander (with surrounding subgerminal ooplasm), artificially placed in contact with Rauber's sickle material or sickle endoblast in culture, can function as a substrate for cellular proliferation with again inducing and/or regenerating capacities in the neighboring upper layer [56] (Figs. 12A, 12B).

Even activation of chicken embryo formation can occur by unfertilized quail blastodiscs [57]. In situ on the egg yolk ball, at the end of the intrauterine period and also shortly after laying, the Nucleus of Pander and endophyll are in very close contact with the upper layer. In the Nucleus of Pander, we have observed the presence of some round Feulgen-positive spherules [58].

After [^{3}H]thymidine, [^{3}H]tyrosine, [^{3}H]uridine, or [^{3}H]tyrosine administration *in ovo*, before laying, an intense incorporation of these precursors was observed in/and around the Nucleus of Pander, (Fig. 13) which indicates DNA, RNA, and/or protein synthesis, perhaps in relation with the inductive influence emanating from the Nucleus of Pander. The aspect and evolution of the Nucleus of Pander during early incubation was studied by magnetic resonance imaging [59], however, without histological data.

Caudal marginal zone

The caudal marginal zone contains part of the upper layer of the area pellucida just peripheral to Rauber's sickle (Fig. 3C), below which the first blood islands will develop, after incubation, under influence of the Rauber's sickle [60, 61]. We observed that in the presence of Rauber's sickle but in the total absence of the caudal marginal zone and area opaca a normal embryo (gastrulation and neurulation) can be induced in the isolated central blastodermal area [62]. This demonstrates unequivocally that Rauber's sickle and not the caudal marginal zone is the early organizer of the avian blastoderm [36]. In any part of an isolated central blastoderm fragment a (caudal) marginal zone (with blood island formation) can be evoked by placing Rauber's sickle material on its deep side. Thus Rauber's sickle not only organizes the upper layer of the area centralis (intramuros) but also the upper layer of the peripheral marginal zone (extramuros).

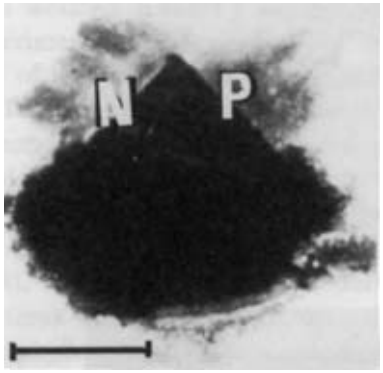


Fig. 12A. Isolated caudal quadrant of unincubated quail blastoderm from which the endophyll was removed, and on which instead a quail Nucleus of Pander (NP) and surrounding subgerminal ooplasmic mass was placed, at the start of *in vitro* culture. Bar = 1 mm.

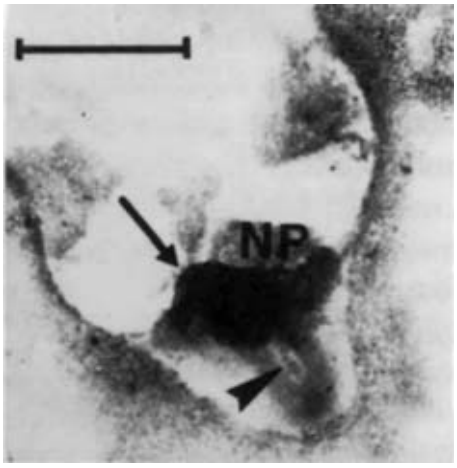


Fig. 12B. The same after 24 h of culture. A normal miniature embryo is visible with a head fold (arrow) and primitive streak (arrowhead). Remnants of Nucleus of Pander (NP) are still present. Note that the surface area of the embryo has not increased. Bar = 1 mm.

Area opaca and subgerminal peripheral ooplasm

Below the upper layer of the area opaca a peculiar peripheral subgerminal ooplasm is found [58]. After *in toto* Feulgen staining, large numbers (250 per mm²) of 40-µm diameter clusters of grouped mitotic figures are seen. After *in ovo* application of [³H]thymidine, [³H]uridine, or [³H]leucine, also an intense labeling of these sub-blastodermic chromosome groups and surrounding intervitelline

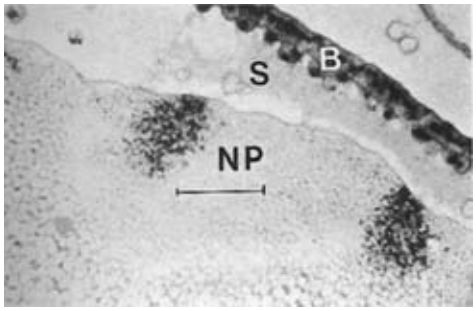


Fig. 13. Autoradiograph of a section through a prelaid quail germ incubated in albumen containing ³H-thymidine: intense labeling in finely granular form in/and around the Nucleus of Pander (NP); B: blastoderm; S: subgerminal cavity. Bar = 100 µm.

material was seen (Figs. 14, 15). The function of these elements localized in a giant annular syncytium of the area opaca, is not known. After total excision of the area opaca with Rauber's sickle still *in situ*, a primitive streak embryo will develop in the area centralis after culture. Blood islands are seen in the neighborhood of the Rauber's sickle but no vitelline blood circulation and no heart appear and the embryo finally dies.

After local excision of the area opaca, blood vessels are only absent in the involved area (unpublished results). This indicates that the area opaca must be considered as an indispensable part of a mosaic embryo, perhaps as the result of a peculiar chromosomal configuration in its subgerminal ooplasmic syncytium.

Endophyll

The endophyll layer (δ-ooplasm-containing, derived from the caudal superficial part of the Nucleus of Pander) is not present in the whole deep part of the blastoderm, as was supposed by Vakaet [16]. By trypan blue induced fluorescent labeling of the δ-ooplasm, we could demonstrate that the endophyll layer in unincubated chicken blastoderms is only present in the caudocentral deep layer region, fixed to the cranial border of the Rauber's sickle (forming a sharp edge visible from the surface of the unincubated blastoderm). Trypan blue-induced fluorescent labeling of the δ-ooplasm confirms the old delamination theory of Pander [5] and Von Baer [15]. Now we presume

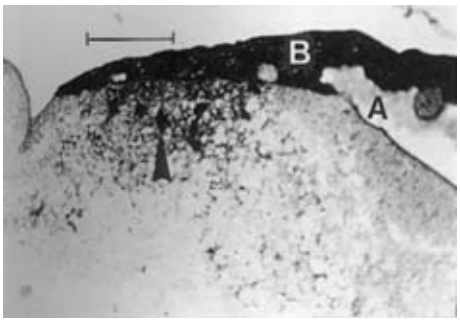


Fig. 14. Autoradiograph of a section through the caudal part of a blastoderm *in situ* on its egg yolk ball incubated in ^{3}H-leucine – containing albumen: intense labeling pattern on the subgerminal chromosome groups (arrowhead) and surrounding intervitelline material under the rim of the blastoderm (area opaca); Note absence of labeling outside the rim of the germ; A: caudal axilla of subgerminal cavity; B: blastoderm. Bar = 100 µm.

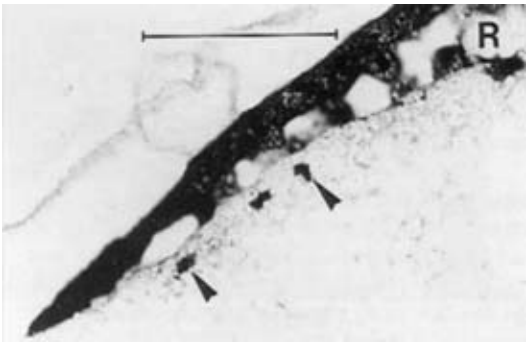


Fig. 15. Autoradiograph of an unstained section through the cranial part of an extracted quail germ, 10 h after the injection of 100 µCi^{3}H-uridine in the surrounding albumen. The subgerminal chromosome groups (arrowheads) incorporate intensely the precursor; R: cranial recess of subgerminal cavity. Bar = 100 µm.

that endophyll cells segregate from the caudal region of the upper layer by polyingressions. We found thus no evidence that upper layer turns inwards below the rim of the blastoderm forming endophyll as was supposed by Vakaet [20]: the so-called migration theory. The origin of endophyll is thus completely different from the origin of the sickle endoblast which is derived from the Rauber's sickle (γ-ooplasm). Vakaet obviously also considered the deep cranial fragmentary anti-sickle region as belonging to the endophyll. Endophyll placed *in vitro* on the deep part of an isolated anti-sickle region (where only upper layer is present) induces a preneural plate with groove [63]. This occurs also during normal development by displacement of the endophyll, pushed in a cranial direction by the cranially expanding sickle endoblast (Figs. 9, 10B). A preneural plate can also be obtained by placing an early Nucleus of Pander, removed from an extracted prelaid egg, on the deep part of an isolated anti-sickle region. Thus the initiation of neurulation (preneurulation) can occur in the absence of gastrulation phenomena. Endophyll orients the direction of the primitive streak and later the head region [37]. Indeed after placing an endophyll sheet on the upper layer of the area centralis at the inner side of a sickle horn (Fig. 16A), we could obtain the formation of two primitive streaks which progressively fuse in their middle region and form conjoined twins (parapagus) (Fig. 16B) [64].

In contrast, after placing a quail Rauber's sickle on the anti-sickle region of an unincubated chicken blastoderm (Fig. 17A) and 20 h culture, head against head twins are induced in one and the same chicken upper layer (Fig. 17B). The head of both twins is directed centrally, where endophyll is present.

Primordial germ cells

Because PGC's are found close to the endophyll and seem to immerge from it, Vakaet [23] supposed that they were derived from the endophyll. Dubois [65, 66] also concluded that the endophyll is at the origin of the formation of PGC's in birds. In general it was accepted that chicken germ cells originate from the deep layer. By contrast Eyal-Giladi *et al.*, [67] concluded by using chick-quail chimeras, made just before primitive streak formation, (i.e. stage XIII: 10-12h incubation) that avian PGC's were from epiblastic origin. Avian PGC's where then thought to arise through a gradual epigenetic process. However in these older blastoderms, the deep layer is no longer composed of endophyll but mainly formed by sickle endoblast, derived from Rauber's sickle. Indeed the endophyll and associated PGC's are then already displaced cranially and adhere to the deep cranial part of the epiblast and to the hemicircular fibrous bands there [68]. By using

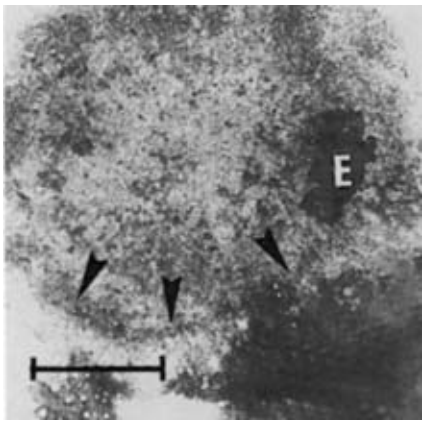


Fig. 16A. Unincubated chicken blastoderm. A fragment of quail endophyll (E) has been placed at the inner side of the left sickle horn. Bar = 1 mm.

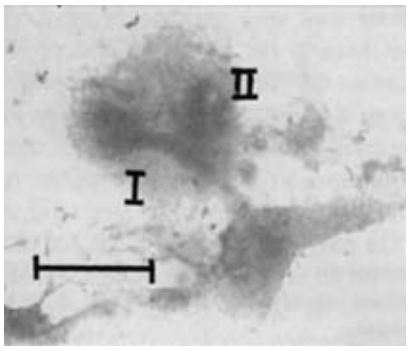


Fig. 16B. The same after 25 h of culture; two conjoined twins, formed by fusion, are visible; two head regions (I and II) are obvious. Bar = 1 mm.

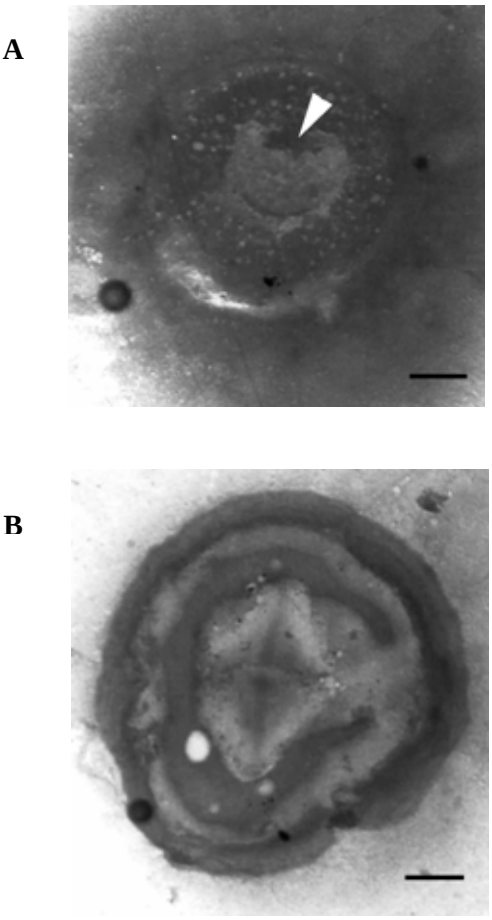


Fig. 17. Artificial production of avian twins by the inductive effect of Rauber's sickle material: **A**: A massive quail Rauber's sickle fragment (arrowhead) is placed in culture on the anti-sickle region of an unincubated chicken blastoderm; note the narrow autochthonous chicken Rauber's sickle. Bar = 1 mm. **B**: The same blastoderm after 20 h of culture: two head against head twins (one induced by the quail Rauber's sickle fragment and the other by the autochthonous chicken Rauber's sickle) have formed in one and the same chicken upper layer. Bar = 1 mm.

trypan blue induced fluorescent labelling of the yolk layers of quail oocytes during their final post-lampbrush stage, we could demonstrate that primordial germ cells together with the endophyll contain yolk from the deep central region of the germ disc i.e. δ-ooplasm from the superficial part of the Nucleus of Pander [69, 55]. So nearly 95 % of PGC's can be labelled 6 - 7 days after one single injection of trypan blue in the mother quail. Oocytal yolk labelling, 1 to 4 days after an injection gives no labelling of the primordial germ cell yolk, but gives labelling of more superficial somatic cells which contain more superficial ooplasms (β or γ). The original deep and central localization of pPGC material has recently been confirmed by the use of a chicken vasa homologue [70]. The data of Callebaut [55] and Tsunekawa *et al.* [70] thus indicate that a kind of preformation may be the mechanism for germ cell specification in birds. In birds there exists thus an early segregation of germ cell ooplasm and somatic cell ooplasm. Our conclusion is in agreement with Wolpert [71] and Extavour and Akam [72] that epigenetic germ cell development is an exception and that most animals use localized ooplasmic determinants to specify the germ line.

Egg white derivatives and permeability of the vitelline membrane: Formation of the subgerminal space, of the paragerminal zone and the epigerminal space

Subgerminal space

During early incubation the albumen loses much liquid, which apparently is transferred from the albumen into the egg yolk ball [73]. This transferred water does not distribute itself evenly through the yolk. Most of it, accumulates immediately below the blastoderm and gives rise to subblastodermic fluid [74]. According to New [75] the most important function of the subblastodermic fluid is to assist respiration (before the chorioallantois has formed) of the blastoderm by pushing the latter very close to the shell-membrane and shell. This upwards pressure makes it very difficult or impossible to avoid damage to the embryonic structures (particularly in the small eggs of some species of birds), when making windows in the shell directly above the embryo. We have described a harmless method where the egg is opened at the diametrically opposite side, by special manipulation of the egg shell and egg contents [76]. From the experiments of New [75] it was not clear whether the blastoderm itself or/and the surrounding vitelline membrane (exterior to the blastoderm rim) enabled transfer of fluid.

Paragerminal zone

We demonstrated by incubating unlabelled embryonated egg yolk balls *in ovo* in albumen (maternally labelled with ^{3}H tyrosine) (Fig. 18) and histoautoradiography that large ^{3}H-labelled clusters of acellular material penetrate through the vitelline membrane, accumulate under it and between the large yolk units exterior to the blastoderm edge [77] (Fig. 19). The largest labelled clusters are usually found close to the blastoderm edge. Further they can be found over a distance of more than 1 mm from the most distal nuclei of the margin of overgrowth. This permeability of the vitelline membrane, of the avian blastoderm can explain some of the *in vitro* experiments of New [75] and indicates that the formation of the subblastodermic fluid is not necessary exclusively due to the secretion by the blastoderm after incubation of fertilized uterine egg

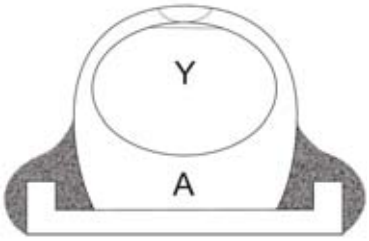


Fig. 18. Drawing of an axial section through a reconstituted quail egg: a nonradioactive fertilized egg yolk (Y) transplanted in maternally labeled radioactive albumen (A).

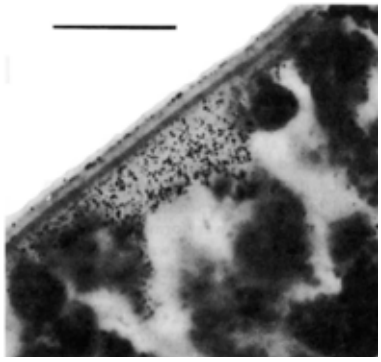


Fig. 19. Autoradiograph of a section through the acellular rim zone, exterior to a quail blastoderm, 43 hr after the beginning of incubation of a non-radioactive egg yolk transplanted *in ovo* in maternally labeled radioactive egg white (as represented in Fig. 18). Note the large cluster of autoradiographic silver grains on the unstained acellular area, pressed between the unlabelled yolk units below the vitelline membrane. The labeling of the narrow film of egg white adhering to the surface of the vitelline membrane is also visible. Scale = 20 µm.

yolks (Fig. 19). We found also autoradiographic evidence for the penetration of radioactively labelled albumen-derived material into the avian subgerminal space, already during the cleavage stage [78].

Epigerminal space

Approximately one day after the beginning of incubation, a space appears between part of the surface of the avian blastoderm and the vitelline membrane. This space has already been observed long ago [5]. In older embryos it will form part of the amniotic cavity. We call it the epigerminal space and we have demonstrated by its intense

radioactive labelling that it is not due to mechanical retraction nor that it is an embryonally derived product but is formed by penetration of egg white derivatives through the vitelline membrane (Fig. 20).

The radioactive labelling of the albumen is performed by injection of ^{3}H-leucine in the mother quail. By dark field illumination of sections through the epigerminal space, it could be seen that it contained a post fixation cast formed by precipitation of its contents. A pronounced labelling was seen in the albumen of the second egg, laid after the injection (Fig. 20). The labelling in the tissues of the blastoderm was much lower. The existence of a pronounced labelling in the two transparent media (albumen and epigerminal space) on each side of the vitelline membrane indicates that the ^{3}H-labelled egg white macromolecules, which contain a substantial amount of leucine can penetrate through the vitelline membrane and are not elaborated by the embryo itself [79].

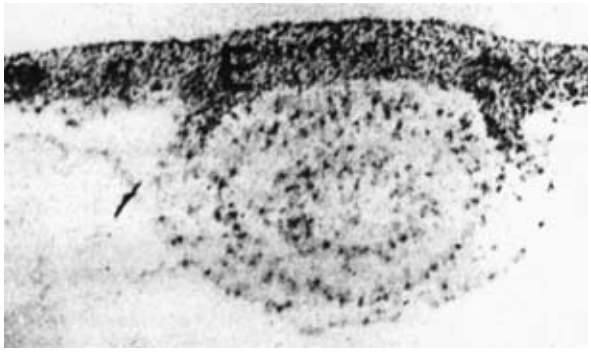


Fig. 20. Transverse section in the cephalic region of a 4 somite quail embryo, developed in the second egg laid after maternal injections of ^{3}H-leucine. Note the intense labeling in the epigerminal space E (X 240).

Comparison of the speed of diffusion of metabolites through the albumen or yolk

By injection of ^{3}H-thymidine into the subgerminal yolk, a distinct concentrated labelling of the blastoderm nuclei could not be demonstrated in chicken germs with less than 500 blastomeres [80]. In contrast we observed a rapid penetration *in vivo* of ^{3}H-thymidine via the albumen layer around quail eggs even during the cleavage stage [81]. Herefore we injected regularly laying Japanese quails intraperitoneally with 1mCi methyl-^{3}H-thymidine. After 2h the eggs were extracted from the uterus by manual expulsion according to Olsen and Byerly [82] and their germdiscs were fixed and examined by histoautoradiography. Two hours after the maternal injection of ^{3}H-thymidine, strong labelling was seen in most of the nuclei or chromosomes of the blastomeres (Fig. 21).

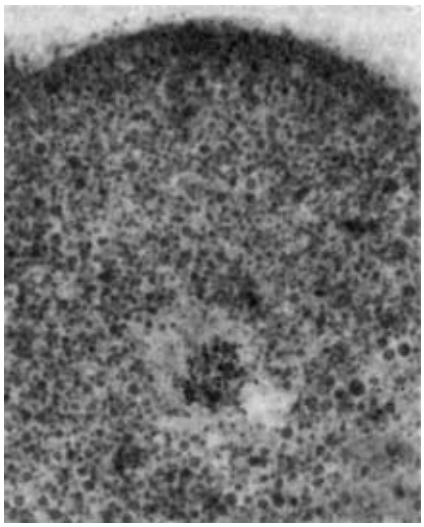


Fig. 21. Autoradiograph of a section through a large blastomere (with labeled nucleus, indicating DNA synthesis) of a quail germ with approximately 32 cells, fixed 2 h after a subcutaneous maternal injection of 1 mCi ^{3}H-thymidine (x660).

The presence of extensive labelling indicated that the radioactive precursor, following circulation in the blood stream, was able to pass through the wall of the uterus into the fluid surrounding the egg and penetrate the shell and shell membrane, albumen and vitelline membrane. The labelling was finally found into the nuclear DNA of the blastomeres during their S period (Fig. 21).This rapid penetration via the albumen way is able to be partially explained by some of the known physical properties exhibited by the albumen in hard-shelled eggs e.g. the rapid diffusion of Bromine 82 [83]. Thus during the early avian embryonal development the metabolites seem to be rapidly taken up from the superficial surrounding structures and not from the yolk mass. In contrast, the intracellular yolk granules

localized in the blastodermal cells are rapidly taken up [84, 85].

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