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An Integrated Experimental Study of Endoderm Formation in Avian Embryos

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Summary. The formation of the endoderm during primitive streak stages in avian embryos was studied by combining several of the following techniques for each embryo. These included microsurgery, time-lapse filming, use of chick-quail chimaeras, tritiated thymidine autoradiography and a novel technique for identifying the morphology of the cells after small pieces of tissue from known areas had been maintained in culture for 24 h.

Using these techniques we have confirmed that the ventral layer of the early chick embryo receives contributions from both the marginal and the central regions of the area pellucida. The former seems to consist of yolky cells derived from the germ wall, whilst the latter consists of smaller, less yolky cells derived from the more dorsal layers of the embryo. The movement of the lower layer anteriorly during these stages appears to be dependent upon mechanical constraints imposed upon it by the expanding tissue in more caudal regions. The extent of each of the two contributions to the lower layer was determined as a function of stage and presence or absence of a lower layer, and the findings are discussed in the light of the existing literature.

Key words: Chick embryo – Endoderm – Primitive streak – Morphogenesis – Germ layers

Introduction

At the time the egg is laid, the chick embryo is composed of a flat disc of cells in which an inner area pellucida and an outer area opaca can be distin-

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guished. After a few hours' incubation an early lower layer forms in the area pellucida ventral to the dorsal layer, the epiblast. Further incubation leads to the formation of the primitive streak in the area pellucida and at the same time a new layer arises between the other two, the middle or mesodermal layer, whilst the early lower layer is progressively replaced by cells which insert into the existing layer, to form the embryonic endoderm or definitive endoblast.

Despite much work in the field since the latter part of the last century, (reviewed by Vakaet 1967, 1970; Bellairs 1971, 1981; Nicolet 1971) the origin of the endodermal layer in the chick embryo is far from understood. It is fairly well established that the definitive endoblast, which will give rise to the embryonic endoderm of the foregut (Bellairs 1953a, b), arises from the overlying tissues. Work which has given support to the epiblastic origin of the embryonic endoderm includes time-lapse cinemicroscopy (Vakaet 1970), marking techniques with carbon or carmine (Hunt 1937; Fraser 1954; Lutz 1955; Spratt and Haas 1960, 1965; Vakaet 1962; Modak 1963, 1965, 1966), use of quail-chick chimaeras (Fontaine and Le Douarin 1977; Vakaet 1973), or tritiated thymidine labelled grafts (Rosenquist 1966; Nicolet 1965, 1967, 1970, 1971; Gallera and Nicolet 1969; Modak 1966). Morphological studies at the electron microscopical level (Vakaet and Hertoghs-de Maere 1973; Wakely and England 1978) showed that the appearance of the endoderm in the centre of the area pellucida (definitive endoblast) is different from the lower layer in more peripheral regions, both in surface appearance and with respect to the cytoplasmic inclusions.

Another major component of the endoderm layer, the 'primary' hypoblast, appears to arise earlier in development, prior to the formation of the primitive streak (see Bellairs 1971, 1981; Nicolet 1971). It is generally assumed that the hypoblast forms by delamination or polyinvagination of cells from the upper layer (Jacobson 1938; Litke 1978; Wakely and England 1978; Fabian and Eyal-Giladi 1981), though there is as yet no direct experimental support for this idea (see Bellairs 1981).

Despite the vast body of data available, most authors have restricted their studies to one technique only and most of the techniques used do not allow the determination of the precise contribution to the lower layer from each of the possible sources of cells. The aim of the present study was therefore to combine a number of techniques to attempt to determine with greater accuracy the extent of each contribution during regeneration and normal development of the lower layer. By using a combination of techniques on each embryo we hoped to overcome many of the shortcomings of each individual technique.

Materials and Methods

Hens' eggs (Ross Rangers) were obtained from Ross Poultry (South) Ltd. and quail eggs from Houghton Poultry Research Station, Huntingdon, Cambridgeshire. The eggs were incubated to the desired stages, between stage XIII (Eyal-Giladi and Kochav 1976) and stage 5 (Hamburger and Hamilton 1951) in a Westernette rotating incubator or a bench top incubator at 38° C for 6 to 24 h.

Surgical operations were carried out on the vitelline membrane under Pannett-Compton saline according to the explantation technique of New (1955), using tungsten needles sharpened with molten sodium nitrite. After the operations, most of the saline was withdrawn from the preparations

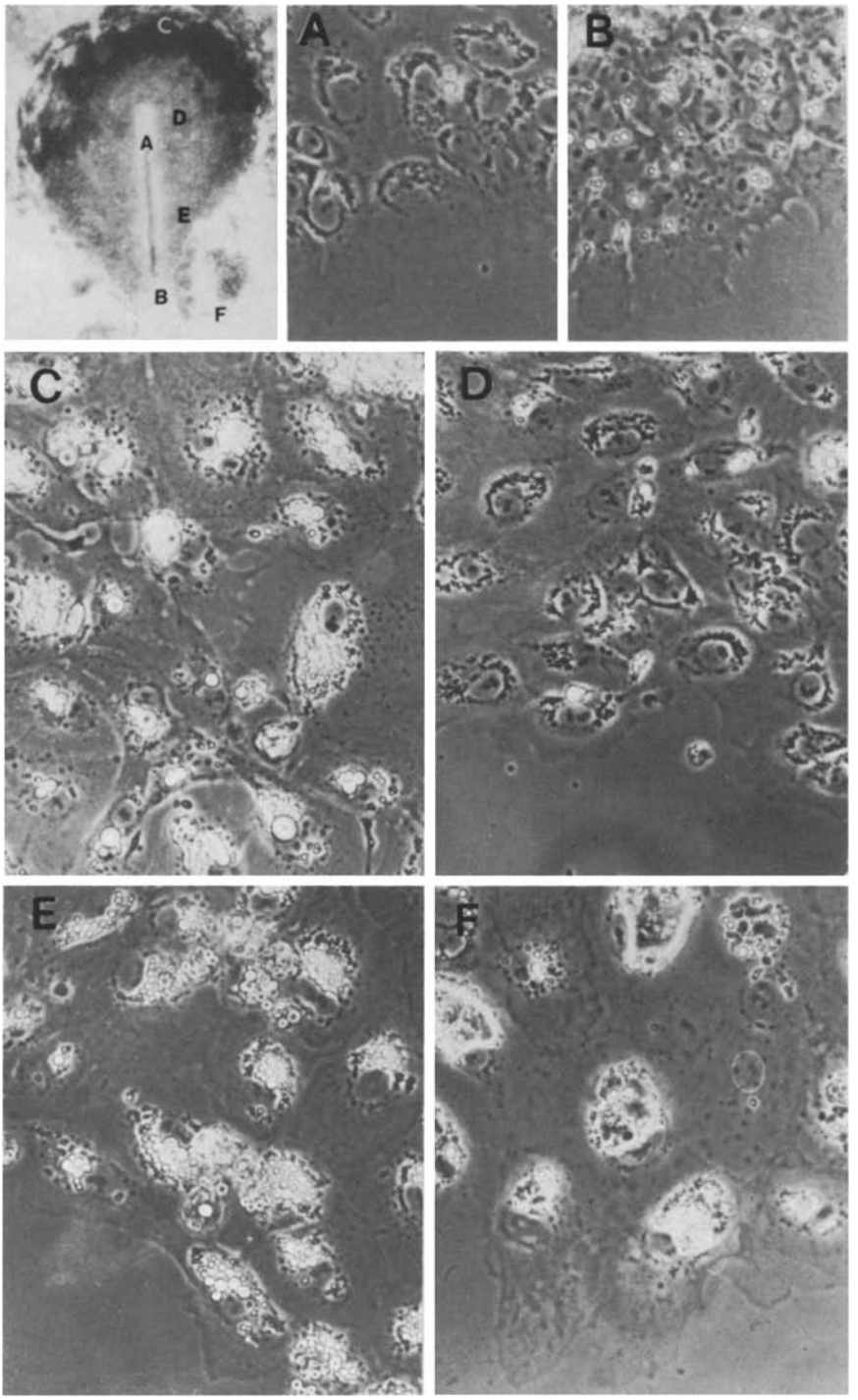

Fig. 1. Explants taken from various regions of a stage 4 chick embryo and cultured for 24 h. The letters on the embryo (top left) correspond to those designating the explants (**A–F**). **C–F** are explants from the lower layer. **A** and **B** are explants from the primitive streak after the lower layer had been removed. (Embryo ×28; explants ×270)

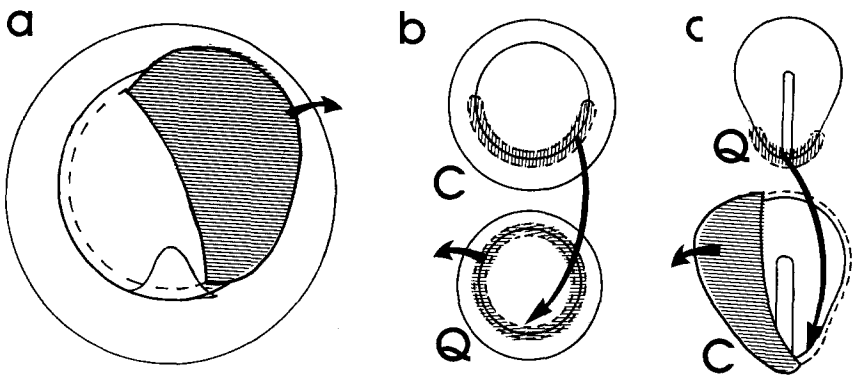

Fig. 2. Diagram of some of the experiments performed. **a** removal of the lower layer (margin left in place) from a stage 2 embryo. **b** posterior margin graft between two stage XIV embryos (in this case chick donor, quail host). **c** posterior margin graft into a host where the lower layer was removed; quail donor and chick host at stage 3

and each embryo was then transferred on its vitelline membrane onto a shallow layer of thin albumen in a Falcon dish. The lid was sealed to the dish with a thin smear of albumen and the dishes maintained at 37° C for about 24 h. Grafting between two embryos was carried out with both donor and host on the same vitelline membrane. The graft was slid to the host with tungsten needles, and then the donor was removed and discarded. Embryos were allowed to heal for a short time at room temperature.

In some cases certain areas of the embryo were marked with carmine (water-insoluble form) using a tungsten needle. This was dipped in the powder and applied gently to the surface of the tissue to be marked after the saline had been withdrawn.

Embryos for histology were fixed in Bouin's fluid or buffered formal saline and sectioned at 5 or 8 μm for staining with Harris' haematoxylin and eosin. To visualise the quail nucleolar marker, embryos were fixed in Zenker's fixative, sectioned at 5 or 12 μm and stained with Feulgen's stain (Le Douarin 1973).

Feulgen's stained sections were photographed with Ilford Pan-F film using a Zeiss PlanApo 40× oil immersion objective (numerical aperture set to 1.0) and a green filter. The film was developed in Acutol for 10 min and printed onto Ilfobrom grade 5 paper to increase contrast. This technique allowed clear visualization of stained nuclei with a minimum of background structures.

Embryos for autoradiography were labelled for 6 h in New (1955) culture in albumen containing 2 μCi tritiated thymidine (^{3}H-TdR) per embryo (Specific activity 5 mCi/mmol. Diluted 1:50 with Tyrodes' saline, then 1:2 with thin albumen). After labelling, the embryos were thoroughly washed using several changes of Tyrodes' solution before grafting into unlabelled host embryos as described above. The resulting embryos were incubated as above, fixed in Bouin's fixative for 24 h, embedded in paraffin wax and then sectioned serially at 8 μm. The sections were mounted onto gelatinised slides (Rogers 1979), dewaxed in Xylene and darkly stained with Harris' haematoxylin. They were then thoroughly washed in running tap water and dipped individually into molten Ilford K2 Nuclear Research Emulsion. After drying, they were kept in black plastic boxes containing silica gel and exposed at 4° C for 4 weeks. They were then developed in Kodak D-19b developer for 20 min, fixed (Amfix, 1:4 for 5 min), dehydrated and mounted in Canada Balsam. The autoradiographs were photographed using dark-field optics.

Explants were taken from the cultured embryos. The embryos were flooded with Tyrodes' or Pannett and Compton saline and small pieces dissected using tungsten needles. The pieces were cultured for 24 h on glass coverslips in glass chambers containing 0.4 ml of medium 199 (Wellcome), foetal calf serum (Gibco) (10%) and Penicillin-Streptomycin (250 i.u./ml, 250 mcg/ml respectively) in an incubator (LEEC) at 37° C in the presence of CO_2. The pH of the medium was 7.4 (see Sanders et al. 1978; Bellairs et al. 1981).

Time-lapse filming was carried out using a Bolex H-16 camera with time-lapse attachments

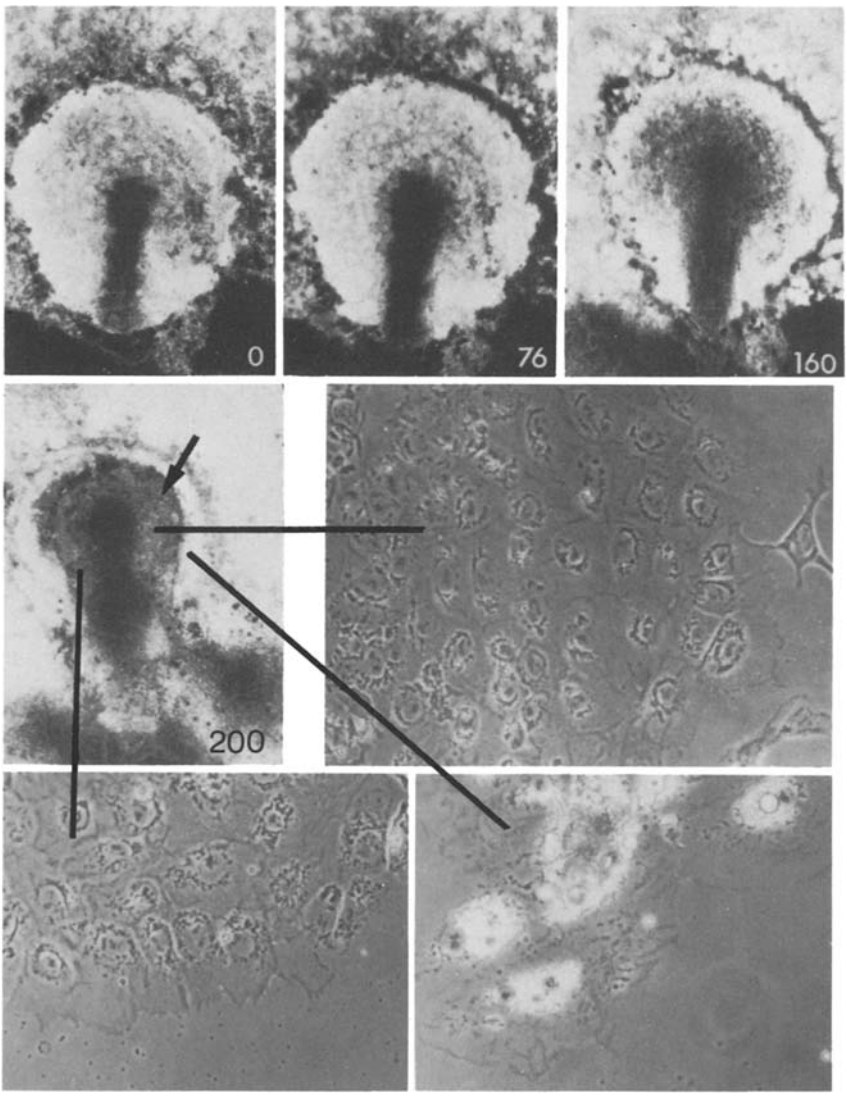

Fig. 3. Formation of a new lower layer. Four frames from a time-lapse film of an embryo developing after removal of the lower layer (margin left in place) at stage 3. The numbers indicate time (min) after the operation. After 200 min explants were taken from the regions indicated in the last frame and grown for 24 h in culture. The embryo was subsequently returned to the filming chamber, filmed for another 10 h or so, and then another set of explants were taken (not illustrated). The lower layer emerging from the primitive streak can be clearly seen in the last frame (*arrowed*). This region contains cells resembling definitive endoblast whilst the more peripheral region contains larger, yolkier margin-derived cells. (Time-lapse frames ×25; explants ×200)

(Wild) fitted to a Zeiss Standard WL microscope. A 1× Plan objective and bright field optics without a condenser was used. Ilford Pan F 16 mm black and white film was used.

A total of over 175 operations were performed on early chick and quail embryos.

The staging system used follows that of Eyal-Giladi and Kochav (1976) with Roman Numerals for pre-primitive streak stages. For the later stages, we have followed those of Hamburger and Hamilton (1951) with intermediate stages designated with + signs.

Results

1. Criteria for the Identification of Tissues

Small pieces of tissue explanted from different areas of the lower layer of the early chick embryo and grown in tissue culture for 24 h could be classified into different morphological categories according to the size and the appearance of the yolk inclusions in their cells. A guide to the criteria used in the present study can be found in Fig. 1.

The largest and yolkiest cells were found in the endoderm of the area opaca or germ wall (F, Fig. 1), in the more posterior regions of the area pellucida close to the margin of this area (E, Fig. 1) and in the anterior germinal crescent (=entophyll crescent or hypoblast) (C, Fig. 1). Cells in central regions of the area pellucida in the vicinity of the anterior tip of the primitive streak were much smaller and contained very small phase-dark cytoplasmic inclusions (D, Fig. 1). In the primitive streak itself, explants taken from anterior and posterior regions after removal of the lower layer differed from each other. The cells from the more anterior explants were similar to those obtained from the definitive endoblast region (c.f. A and D, Fig. 1). The cells from the more posterior explants were smaller still, with very few phase-dark cytoplasmic inclusions, and the submarginal regions of the explants contained numerous cells which were less well spread than any of the other explants (B, Fig. 1). (See Voon (1980) and Ireland et al. (1978)).

2. Complete Lower Layer Removal

The entire lower layer in the area pellucida was removed in 30 chick embryos ranging from stage XIV to stage 5. In 19 embryos the proximal margin of the lower layer (germ wall margin) was also removed, while it was left in place in the remaining embryos (Figs. 2a, 3). Of the 30 only two did not develop further.

Table 1. Results of embryos regenerating a new lower layer after removal of the original lower layer including or excluding the germ wall margin. These are results from 27 chick embryos. Each symbol represents one embryo: +, lower layer present; −, lower layer not present; 0, presence of new lower layer cannot be determined with certainty as the region cannot be dissected, or the explants will not grow in culture. The criteria used for the presence of a new lower layer were: (a) whether or not the region could be dissected, (b) whether a lower layer was visible in time-lapse films, and (c) morphology and behaviour of the explanted tissue in culture

Stage	With margin		Margin removed	
	From germ wall	From streak	From germ wall	From streak
XIV	+++	+++	−−−−	−−−0
2–2$^+$	++	++	−−−−	−000
3–3$^+$	++	++	−−−−−−	+++++0
4–5	++	++	−−−−	++++

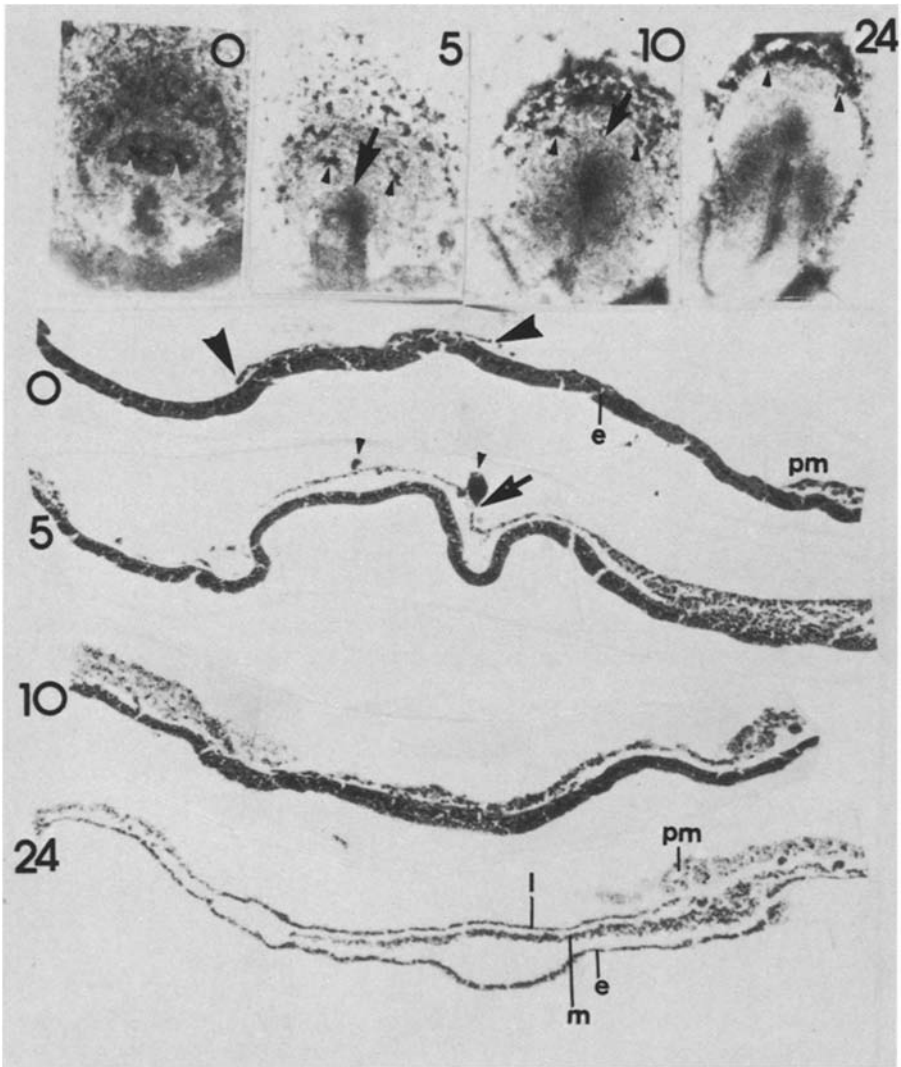

Fig. 4. Partial removal of the lower layer. At the top of the figure there are four frames from a time-lapse film showing an embryo from which the lower layer had been removed except for a small piece anterior to the primitive streak. Note that the movement of the carmine (*arrowheads*) marked piece did not begin until about the time when it was met (*arrows*) by the new lower layer emerging from the primitive streak after 5–10 h. The lower part of the figure shows four saggital sections through similar embryos at corresponding times after the operation (0, 5, 10 and 24 h). The posterior (caudal) end of the embryo is shown towards the right of each section and lower layer uppermost. The position of the remaining piece of the lower layer is marked with large arrowheads. *e* epiblast; *pm* posterior germ wall margin; *m* middle layer; *l* new lower layer. (Embryos ×16; sections ×75)

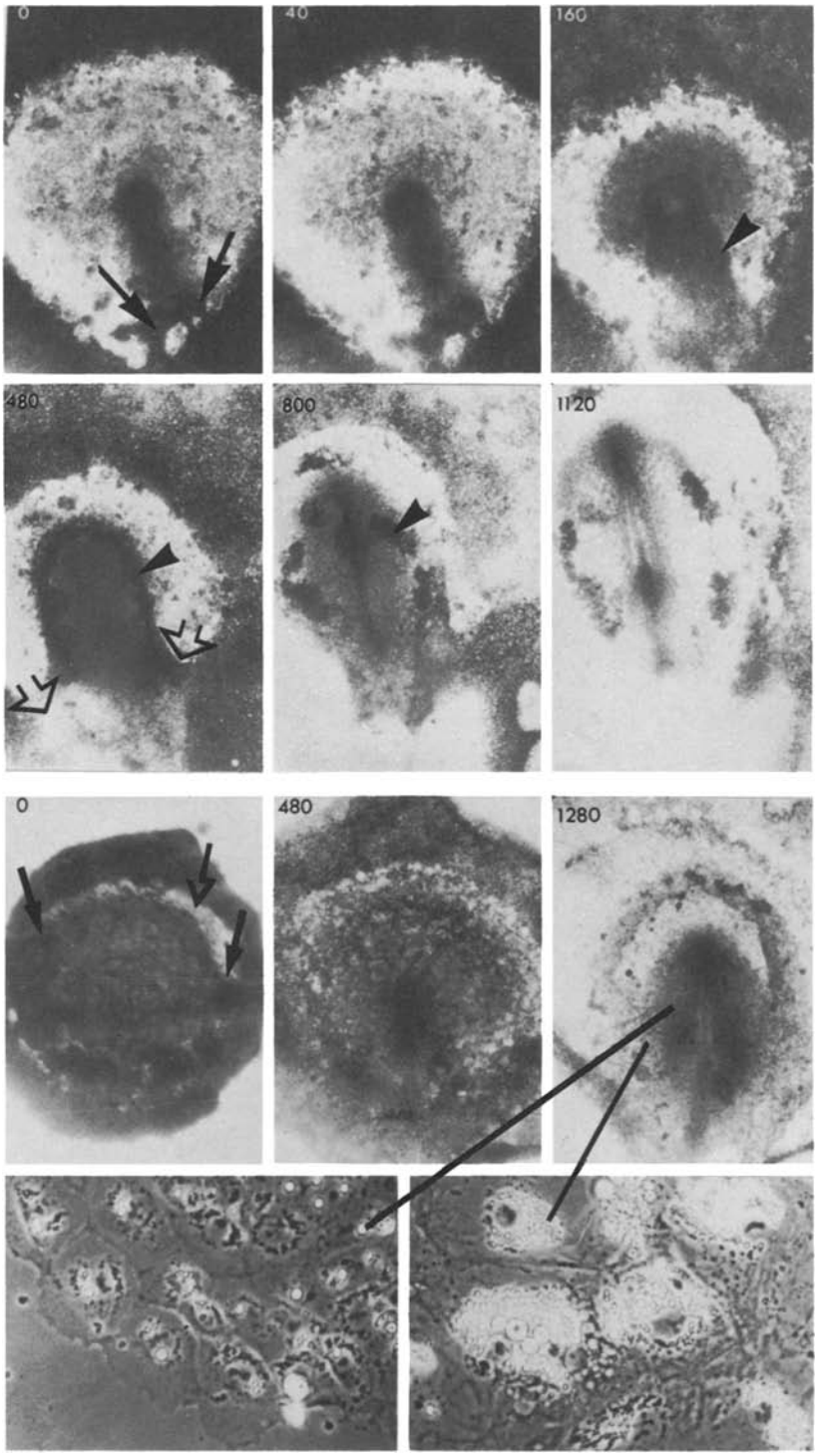
0
40
160
480
800
1120
0
480
1280

Figure 3 shows an embryo deprived of its lower layer at stage 3. The margin was not removed. The new lower layer emerging from the primitive streak can be clearly seen in the time-lapse film. Explants taken from the region of this visible lower layer show small cells with phase-dark inclusions as in normal, unoperated embryos, whilst those taken from more peripheral regions show much larger and yolky cells with a morphology characteristic of germ wall explants.

Table 1 summarises the results obtained from chick embryos from which the lower layer had been removed. It shows that: (a) the germ wall margin, if present, always contributed to the newly formed lower layer; (b) there was a correlation between the presence or absence of this margin with whether or not the new lower layer received a contribution from the primitive streak, and (c) whether or not this contribution took place was also dependent on the age of the embryo.

Four embryos which had been deprived of their lower layer but not their margin were allowed to remain overnight at room temperature. By taking explants from these embryos it was found that some tissue had moved into the area pellucida from the marginal regions, whilst a contribution from the primitive streak could not be demonstrated. None of the embryos had developed a complete lower layer under the area pellucida.

3. Margin Removal

In 7 chick embryos at stage XIV-4 the entire margin of the lower layer was removed while leaving the central regions of the lower layer in place. In all cases embryos developed normally. In 4 of these embryos a gap was still apparent after 24 h between the central lower layer and the germ wall, whilst in the remaining 3 continuity between the two regions had been re-established, seemingly by spreading of the lower layer tissue from both regions. In the 4 embryos where continuity had not been re-established the germ wall appeared to have retracted centrifugally from the remaining lower layer in the *area pellucida.*

4. Partial Lower Layer Removal

In 9 chick embryos at stage 2 to 4 the lower layer was removed except for a small (about 600×250 μm) piece anterior to the tip of the primitive streak

Fig. 5. Margin grafts. **a** 6 frames from a time-lapse film of a stage 3 chick embryo (lower layer removed) grafted with a piece of quail stage 3 margin (*arrow*). The numbers represent time (min) after the operation. Note the continuity which was established (*open arrows*) between the posterior margin and the regenerating lower layer, and the new lower layer emerging all around the primitive streak (*arrowheads*). **b** 3 frames from another time-lapse film of a quail embryo operated as shown in Fig. 2b, where the donor was a stage XIV chick posterior margin. The host lower layer was not removed. The *arrows* show the position of the grafted margin immediately after the operation; the *open arrow* shows the gap remaining at the anterior end. Note that the graft had healed by 480 min after the operation and subsequent development was normal. Two explants taken from the two different regions of the lower layer of the same embryo are also shown. Note the donor margin-derived yolky cells derived from around the periphery (right) and the smaller host primitive streak-derived cells explanted from near the centre of the area pellucida (left). (Time-lapse frames $\times 25$; explants $\times 270$)

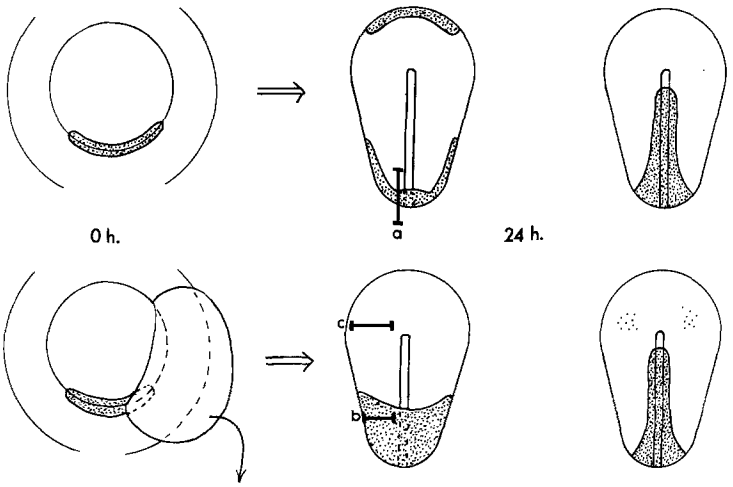

Fig. 6. Summary of results of margin grafts. Diagrams showing the extent of the contribution from a grafted posterior margin with the host lower layer in place (top series) and removed (bottom series). The central diagram in each case represents the extent of margin-derived contribution to the lower layer, whilst the one on the right shows the extent of margin-derived tissue within the middle layer. Only the area pellucida is shown. The levels and letters shown in the middle diagrams correspond to the levels at which the sections in Fig. 7 were taken

which was marked with carmine powder. All these embryos survived the operation. Six of them were fixed at 0, 2, 5, 6, 10 and 18.5 h respectively, while the remaining 3 were fixed at 24 h. The embryos were sectioned parallel and close to the axis of the primitive streak. Figure 4 shows the result of the operations. Time-lapse films indicated that the remaining lower layer piece did not move anteriorly as is its normal pattern of movement (Vakaet 1970) until around the time when the anterior margin of the newly formed lower layer met the posterior border of the remaining original piece.

5. Posterior Margin Grafts

In 13 chick embryos, the entire margin at stage XIV-3 was removed, and a piece of posterior margin from a stage 2–3 quail embryo was grafted posteriorly into the resulting gap of the host. In another 5 embryos the converse (chick margin, quail host) operation was peformed (Fig. 2b). In a further 2 quail and 5 chick hosts the lower layer was also removed in addition to the margin before grafting.

Figure 5a shows a chick host from which the lower layer and margin were removed, and which was grafted with a quail posterior margin. The time-lapse sequence shows the new lower layer which emerged from the host primitive streak after about 160 min, and the continuity which was established between this and the grafted quail margin at the posterior end of the area pellucida. Sections through a similar embryo (Fig. 7b, c) confirmed that the lower layer in posterior

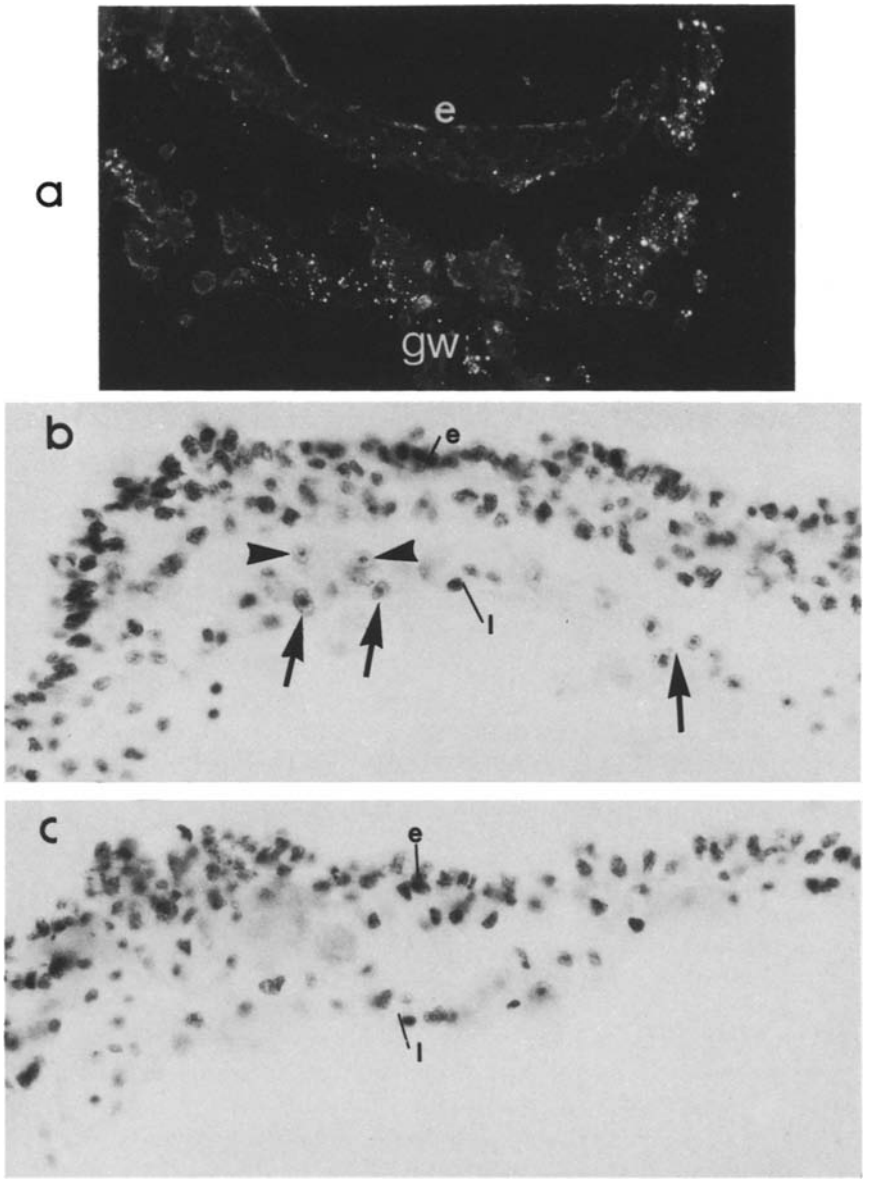

Fig. 7a. Saggital section and autoradiograph through the posterior germ wall region (level a, Fig. 6) of an unlabelled host grafted with a ^{3}H-TdR labelled margin. Note the grains over much of the germ wall endoderm. **b** and **c** are transverse sections through a chick host grafted with a quail posterior margin as described, at levels b and c in Fig. 6. Note the presence of donor quail cells in the lower layer (*arrows*) and middle layer (*arrowheads*) at level b, and their absence at level c. (×180). *e* epiblast; *l* lower layer; *gw* germ wall endoderm

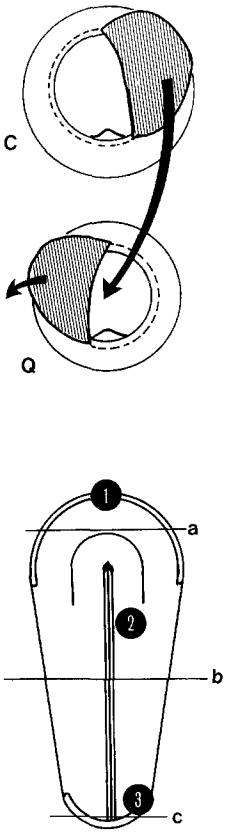

Fig. 8. Whole lower layer grafts. The first diagram schematises the operation (*C* chick; *Q* quail) whilst the second diagram shows the resulting embryo and the levels from which the explants (*1–3*) and sections (a–c) in Fig. 9 were taken

regions of this area contained donor-derived quail cells, the nucleoli of which stain positively with Feulgen's stain. Figure 5b shows a stage XIV quail embryo from which the margin was removed and replaced with the posterior half of the circumference of the margin of a stage XIV chick embryo. The time-lapse sequence shows that the graft quickly heals and subsequent development is remarkably normal. Explants taken from the central regions of the area pellucida lower layer display morphologies consistent with (host) primitive streak-derived cells, whilst those taken from more peripheral posterior regions of the area pellucida lower layer contain cells which are larger and more yolky, consistent with a margin origin.

In most cases of quail posterior margin grafted onto chick hosts, some of the quail cells were found within the host mesodermal layer (see Fig. 7b). This was more common and more extensive in those cases where the lower layer of the host had been removed at the time of the operation (see Fig. 6).

The donor-derived quail cells were rarely found clustered as a region of only quail cells, but were interspersed with a variable number of host chick cells. It was difficult to tell with certainty if this was also the case in the converse operations as it was more difficult to identify a single chick cell in a quail background than the reverse.

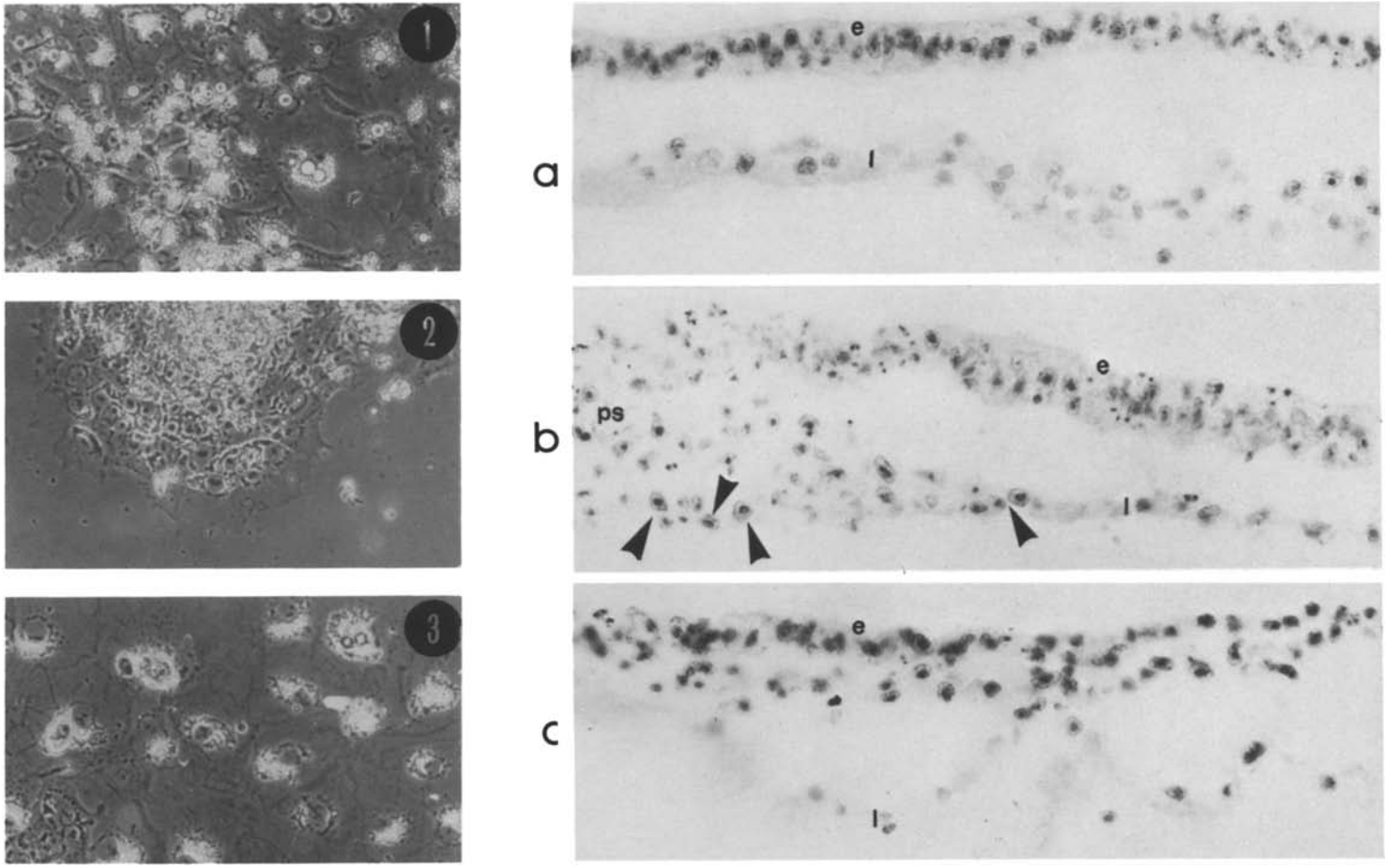

Fig. 9. Whole lower layer grafts. Explants (*1–3*) and sections (**a–c**) at the levels shown in Fig. 8. Note the presence of quail cells in the lower layer (**b**) composed of definitive endoblast-like cells (*2*) in the centre of the area pellucida, and the entirely graft-derived chick lower layer composed of larger, more yolky cells at the extreme caudal and cephalic ends of the area pellucida (**a**, **c**; *1*, *3*). (Explants ×180; sections ×180) *e* epiblast; *l* lower layer; *ps* primitive streak

Figure 6 summarises the results of the various types of margin grafts. It shows that the contribution of the grafted margin to the lower layer is more extensive if the host layer has been removed.

The grafted margin appears to allow for a contribution of the primitive streak-derived tissue to the newly formed lower layer in embryos operated prior to stage 3, where this would have been prevented had a margin not been present (see above and Table 1).

In 5 chick embryos the margin was removed (including the lower layer in 2 of them) and replaced with a posterior margin from tritiated thymidine labelled donor chick embryos as described in the Methods. Autoradiography of histological sections was combined with an analysis of cell morphologies in explants, and the results (Fig. 7a) are in agreement with those obtained using the quail-chick chimaeric marker. The autoradiographs were, however, more difficult to interpret than the latter due to some diffusion of the label into more dorsal layers.

6. Complete Lower Layer Grafts

These experiments were designed to test for a contribution from the primitive streak when a lower layer is present.

In 8 blastoderms of quail, the entire lower layer was removed and replaced with an entire chick lower layer. Donors and hosts were at stage XIV or at stage 2^{+}–3. Figures 8 and 9 summarise the results of these experiments. The explants (1–3) show that as in normal embryos (c.f. Fig. 1), central regions of the area pellucida lower layer contain small cells with phase-dark inclusions, whilst more peripheral regions contain much larger and yolkier cells. The sections (a–c) show that central regions of the lower layer contain host quail cells (Fig. 9b) whilst more anterior and posterior regions contain the grafted chick tissue (Fig. 9a, c).

As in the case of the posterior margin grafts, (see section (5) above), in most cases the cells of host quail and donor chick origin appeared intermixed to some extent, although the predominance of one or another cell type was clearly visible in each region.

These experiments show that a contribution from the primitive streak can take place even when a lower layer is present.

Discussion

Lower Layer Regeneration

Our results show that (a) the newly formed lower layer can receive contributions from both the marginal regions of the *area pellucida* and from primitive streak-derived material. (b) if the margin is present, it always contributes to the new lower layer whilst the extent of the central contribution depends upon the presence of the margin or a lower layer and the precise stage of the operation.

The centripetal movement of margin-derived tissue resembles the spreading of germ wall endoderm and other tissues in culture (see Bellairs et al. 1981).

This centripetal movement begins earlier after lower layer removal than the contribution from the primitive streak, at least in the younger embryos (until stage 3^+). The contribution from the marginal regions does not appear to be inhibited at room temperature unlike that from the primitive streak, which suggests similarities with a previously described wound healing response (Mareel and Vakaet 1977; England and Cowper 1977). This is also not inhibited by room temperature, and appears to consist of a flattening of the cells not unlike that taking place during spreading in culture.

The exact processes leading to the contribution of primitive streak-derived material to the new lower layer appear to be more difficult to understand from our experiments. England and Wakely (1978) have suggested on the basis of SEM observations that the newly formed endoderm is a result of an adaptation of the mesoderm under the operated regions. They observed that the new lower layer never extended more peripherally than the distal margin of the mesoderm. These observations, however, do not rule out other possibilities, for example, that the mesoderm may be required as a substrate for further tissue coming out from the primitive streak. Alternatively, there may be a population of endoderm cells within the middle layer, as the authors suggested. The primitive streak-derived tissue which is to form the new layer appears to consist of such translucent, flattened cells, that it has been impossible for us to establish the mode of incorporation of these cells into the lower layer by means of time-lapse filming.

The observation that either a margin or a lower layer are required to permit a contribution from the primitive streak-derived material could suggest that the advancing edge of the margin-derived material must have reached at least the edge of the mesoderm before any contribution from streak-derived tissue can take place. This is supported by the observation discussed above that the marginal contribution is observable earlier than that from the primitive streak. We have often observed, in time-lapse films of marked embryos, a movement of middle layer tissue into the primitive streak region in the first hour or so after the operation. This was followed by an emergence of mesoderm out of the primitive streak region some time later. It is thus possible that in embryos further developed than stage 3^+ or so, when a considerable amount of mesoderm has already emerged from the primitive streak, the mesoderm cells lying more ventrally might flatten to form a new lower layer as suggested by England and Wakely (1978). In embryos operated prior to stage 3 however, there may be a contribution from the primitive streak cells in the form of newly emerged material. That there is a fundamental difference between the behaviour of operated embryos prior to and after stage $3\text{-}3^+$ is illustrated by their response to the presence or absence of the margin in terms of primitive streak-derived contributions (see Table 1).

The Margin Contribution

Our results show that the presence of a margin is not essential for normal development after the formation of the primitive streak provided that a lower layer is present in more central regions. The presence of a lower layer, however,

appears to be required for a contribution from primitive streak-derived tissue to the lower layer in embryos operated prior to stage 3^+. These observations imply that at least after the 'induction' of the primitive streak at about stage XIII (Azar and Eyal-Giladi 1979), the main role of the marginal region is to ensure the presence of a lower layer under the primitive streak which appears to be required for the streak-derived endoblast to form. Our results are in agreement with those of Azar and Eyal-Giladi (1979), who found that pre-streak blastoderms deprived of the hypoblast and marginal regions did not continue to develop normally. They are also in agreement with those of Bellairs et al. (1967), who established that removal of a ring of area opaca surrounding the area pellucida at stage 4 or so did not prevent normal development.

Our results further indicate (Section 5) that at least some of the middle layer material may arise prior to primitive streak formation and be found in close association with the posterior margin of the area pellucida. Thus the marginal region endoderm appears to contribute not only to the endoderm of the area pellucida, but also to the middle layer of this region (Fig. 6).

Jacobson (1938) was the first to observe a movement of tissue from the posterior regions of the blastoderm into the area pellucida endoderm by using several marking techniques. He claimed that these cells arose from the epiblast by invagination at the posterior margin through a structure which he likened to the archenteric canal of other vertebrate embryos. The techniques available at that time, however, make it very difficult to determine with certainty the origin and destination of the cells which he observed.

Vakaet (1970) described a movement of tissue from the marginal region into the area pellucida as the first visible morphogenetic movement in his time-lapse films of the lower layer. He concluded that the marginal region is the origin of what he terms the 'junctional' endoderm. Vakaet (1970) makes a distinction between this endoderm and 'sickle' endoblast, which also appears to move centripetally from the marginal region, but mainly in more posterior areas; he surmised that the sickle region receives a direct contribution from the epiblast by poly-invagination. We have been unable to distinguish between these two types of endoblast from our experiments. Both Vakaet's and our observations, however, suggest that the margin contributes to the lower and middle layers even when the lower layer is present (Fig. 6), and not just as a wound healing response to removal of this layer. The extent of the contribution from the margin, however, appears to be much greater if the lower layer has been extirpated. This suggests that the presence of a lower layer may introduce a mechanical constraint to the spreading-like movement of marginal tissue centripetally during normal development, which may also contribute to determine the movement pattern of the whole lower layer.

Lower Layer Movement Pattern

We have found that the anteriorly directed movement of the lower layer in regions anterior to the tip of the primitive streak appears to depend upon the presence of a continuous lower layer in more posterior regions. We have also observed that during regeneration as well as during normal development,

the definitive (streak-derived) endoblast progresses outwards from the primitive streak.

Spratt and Haas (1965) claimed that there was no contribution to either the mesoderm or the lower layer by the epiblast by direct invagination through the primitive streak, and that there was no evidence for movement of epiblast into the primitive streak. More current views (see Vakaet 1967, 1970; Bellairs 1981; Nicolet 1971) have concluded, however, that the epiblast does move into the primitive streak, and that this tissue then moves out of the primitive streak to form the mesoderm and the definitive endoblast. The pattern of resulting lower layer movements described by Spratt and Haas (1960) and by Rosenquist (1972) are generally accepted.

From the studies in the literature and from our own observations from grafting experiments and time-lapse films we can conclude that the lower layer after the formation of the primitive streak moves by a combination of two main components: (a) the centripetal addition of new tissue from marginal regions, particularly at the posterior end of the area pellucida, (b) the central addition of new tissue at the primitive streak or immediately adjacent regions. The tension exerted by the expanding edge of the blastoderm is also likely to be an important factor as investigated by Bellairs et al. (1967), New (1959), Downie (1975, 1976) and Chernoff and Overton (1977).

Our finding that cells of donor origin are frequently interspersed with some of host origin in various grafting experiments suggests that there may be a great deal of flexibility in the movement of individual cells within the lower layer. Preliminary films of embryos at these early stages of development taken at high magnification in our laboratory suggest that cell movement within the lower layer may indeed be more stochastic than hitherto supposed (unpublished observations). We are, however, still attempting to resolve some of the optical difficulties of the preparations.

In blastoderms prior to and during the early stages of primitive streak formation, the marginal regions of the area pellucida appear to move in an antero-posterior direction closely following its edge. This "Polonnaise" movement has been studied by Spratt and his collaborators (see Spratt and Haas 1960), Lutz (1955) and Vakaet (1970). We have also observed these movements in our time-lapse films.

Conclusions

The present study, making use of several techniques in combination on the same embryos, has given support to the notion of a dual origin of the lower layer in early avian embryos both during normal development and during regeneration of this layer. The lower layer thus forms partly from a contribution from the epiblast, and partly from a contribution from the margin of the area opaca. We have also established that the presence of the lower layer is required for the formation of the primitive streak-derived definitive endoblast prior to stage 3 and that the main roles for the marginal region are to ensure the presence of this layer during development and regeneration and to introduce some mechanical constraints to the movement of the lower layer after formation of

the primitive streak. The contribution of the marginal region to the lower layer present 24 h after the operation was more extensive if the original lower layer had been removed.

Our study further indicates that the middle layer may also have a dual origin, arising partly from the epiblast via the primitive streak and partly from the marginal region, even prior to the formation of the primitive streak.

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