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From Cleavage to Primitive Streak Formation: A Complementary Normal Table and a New Look at the First Stages of the Development of the Chick

I. General Morphology

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A manual method has been developed to extract eggs from the genital tract without harming the hen. Fourteen developmental stages preceding Hamburger and Hamilton's stage 2 have been studied from live material and photographed from both upper and lower surfaces. Three developmental periods have been distinguished: cleavage, formation of area pellucida, and hypoblast formation. The last two are morphogenetic periods which follow the determination of the embryonic axis. During cleavage the diameter of the germ becomes reduced and the cytoplasmic mass becomes thicker. Later there is a progressive increase of the germ's area. The formation of the area pellucida is a result of an oriented massive loss of the yolk laden cells of the lower layers. The formation of the primary hypoblast is a result of posterio-anteriorly oriented polyinvagination and a later coalescence of the cell aggregates in the same direction. It is not yet possible to conclude whether there is also an anteriorly directed cell movement. The inconsistent usage of the terms blastodisc and blastoderm is discussed. The authors suggest avoiding the term blastodisc and using the general term germ for all the early stages. The term blastoderm may be applied from stage VI onward.

INTRODUCTION

The study of the embryonic development of the chick has been limited for many years almost entirely to the stages from the unincubated blastoderm onward. The reasons for this have been the difficulty of obtaining younger material, for which it was believed that hens had to be sacrificed (Patterson, 1910) and the lack of appreciation of the importance of the earlier stages in embryonic development.

Furthermore, most of the experimental work with chick blastoderms has been performed on embryos from the primitive streak stage and older, on the assumption that the morphogenetic period started with the formation of the streak. This latter assumption was based on the homology which was drawn between the primitive streak and the amphibian blastopore. Development prior to gastrulation in amphibians (or any holoblastic egg) was regarded merely as cleavage, during which period it was generally accepted that "the constituent parts of the cytoplasm of the egg are not displaced to any great extent and remain on the whole in the same positions as in the egg at the beginning of cleavage" (Balinsky, 1970). Thus, before P.S. formation, the chick embryo has been regarded as being in the cleavage period and in possession of no significant morphogenetic activity. Hamburger and Hamilton (1951) have obviously prepared their normal table of the chick to answer to the common needs of most investigators of chick development. It is probably therefore that their stage 1, called prestreak or an unincubated blastoderm, is represented as a disk with no specific details. Their stage 2 blastoderm already contains an initial primitive streak.

The above approach overlooked many known facts concerning the early develop-

[1] Killed in action, October 16, 1973.

ment of the chick embryo. There has been work done on the fertilization and early cleavage in the pigeon (Harper, 1904; Blount, 1909; Patterson, 1909) and in the hen (Goette, 1874; Duval, 1884; Kionka, 1894; Patterson, 1910; Olsen, 1942; Bekhtina, 1960; Emanuelsson, 1965). Some additional work has been done on the formation of the area pellucida (Clavert, 1962) and on the formation of the two-layered blastoderm (Oellacher, 1869; Balfour, 1873; Kolliker, 1875; Koller, 1882; Merbach, 1935; Jacobson, 1938; Peter, 1938; Pasteels, 1945; Spratt and Haas, 1961, 1965; Spratt, 1960; Vakaet, 1962.

However, no coherent picture has been formed to cover the sequence of developmental events from the first cleavage up to primitive streak. The aim of the present study is to fill this gap by presenting a series of normal stages with clear morphologic criteria serving as easy reference points for any investigation on the early stages.

MATERIALS AND METHODS

A technique has been devised to extract eggs mechanically from the hen's genital tract. Hens, either Leghorn or hybrid New Hampshire × Leghorn, were kept in individual cages and artificially inseminated. Prior to a planned extraction of eggs the hens were observed carefully for 1–2 days, and the exact time of laying was recorded for each hen. The time for the first cleavage has been predicted as 6 hr (Patterson, 1910) and 5½ hr (Olsen, 1942) after the laying of the previous egg. Both of these authors report that cleavage begins in the oviduct, but Olsen mentions that it was possible by injecting posterior pituitary extract to obtain eggs 5 hr after the estimated time of ovulation, i.e., 5½ hr after the laying of the previous egg. Olsen and Byerly (1932) describe a method of expelling eggs manually from the hen's uterus, probably at a much later stage. This prompted us to try manual extraction at different times, starting 5½ hr after the laying of the previous egg.

Extraction of the egg was done as follows: First, the hen was checked for the presence of an egg by inserting a finger into the cloaca. Beginning 5½ hr after the laying of the previous egg, the new egg could be palpated in a deep position, probably at the lowest part of the isthmus on transition to the uterus. An egg that could be palpated usually could be expelled manually. The hen was placed on its right side with its left leg held by the left hand of the operator. External pressure was applied with the right thumb to the abdomen anterior to the egg in order to shift it caudally. A second person held the hen by its right leg and caught the egg as it was expelled. This method enabled us to obtain hundreds of eggs at all the desired developmental stages without sacrificing the mothers. The youngest eggs had only a shell membrane, while older ones showed progressive formation of a calcareous shell, the thickness of which was proportional to the duration of its stay in the uterus. The uterine eggs, however, form only part of the material covered in this study; the later stages included in the study are normally laid, unincubated eggs and eggs incubated for various short periods at 37°C. Special attention has been paid to the unincubated eggs, which were collected at the moment of laying and either opened immediately or rapidly cooled and kept at 15°C before being examined.

The eggs were opened into a bowl containing Ringer's solution, the future posterior side of the germ was marked with carbon according to Von Baer's law, and the germ was then dissected out, thoroughly cleaned from adherent yolk, freed of the vitelline membrane, and photographed with a camera mounted on a dissecting microscope. Generally fixation was not employed, but at later stages of development (stage X and onward) a few drops

of formalin were sometimes added to prevent the thinned-out blastoderms from curling. Each germ was photographed both from its upper and lower sides against a black background.

RESULTS

Hundreds of eggs collected according to the above system included all developmental stages. The stages were defined according to distinct morphological criteria and not to hours of development. The time intervals between the stages are not constant and the approximate uterine age is referred to for each stage. The uterine age was calculated as the interval between the laying of the former egg and the extraction of the studied egg minus 5½ hr. Fourteen stages were defined and described; they encompass three distinct developmental periods: A, cleavage period; B, formation of the area pellucida-period of symmetrization; C, period of hypoblast formation. Roman letters have been used in order to distinguish them from Hamburger-Hamilton.

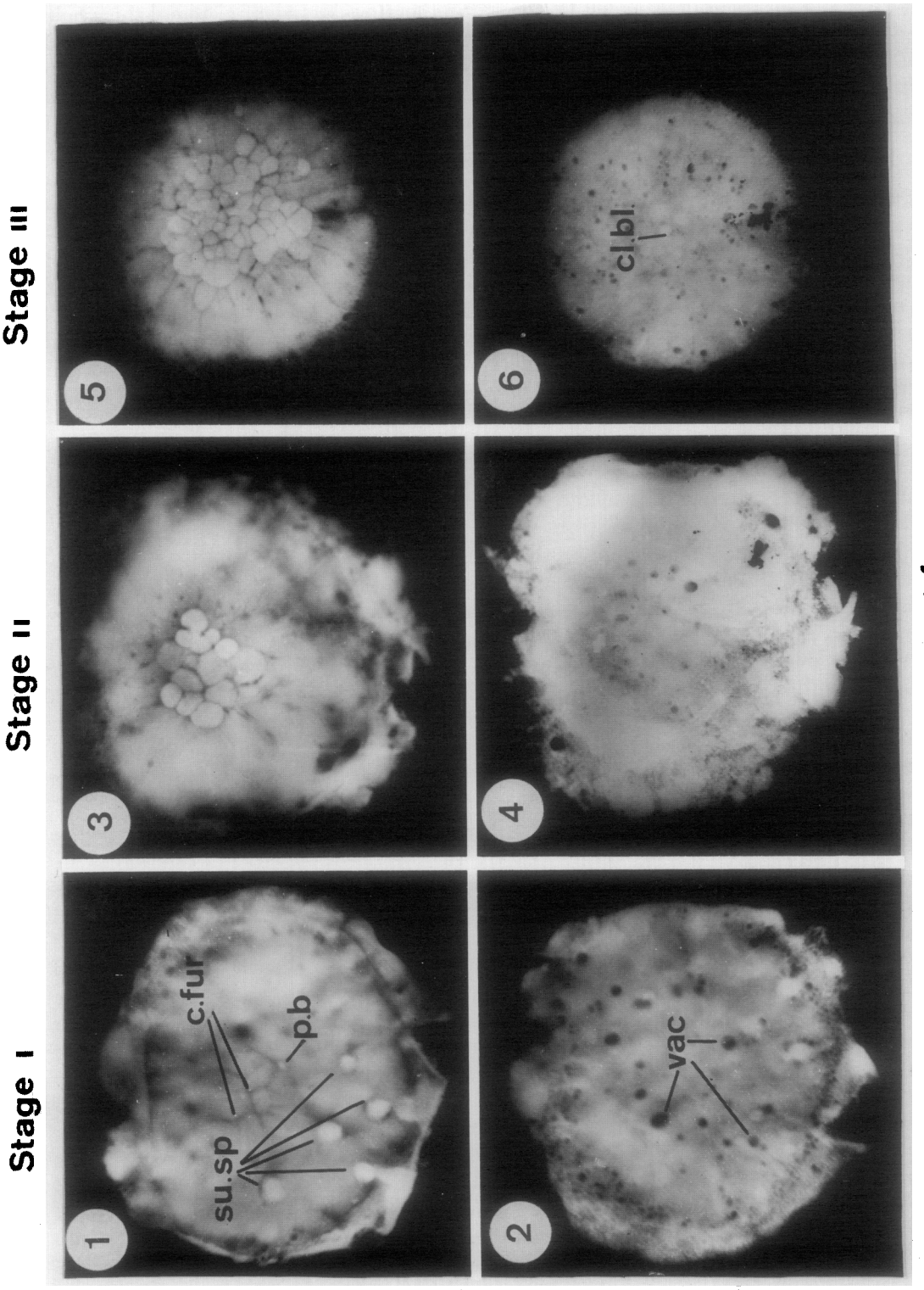
Period A: Cleavage (Stages I-VI)

During this period, the cytoplasmic mass of the germinal disc is cleaving very rapidly, the time span between stages I and VI being about 10-11 hr.

Stage I (0-1 hr uterine age; Figs. 1 and 2). Eggs containing embryos of this stage were extracted from the hens 5½-6½ hr after the former egg had been laid. They were surrounded by a very flaccid shell membrane; the albumen inside the membrane which had not yet absorbed uterine fluid was very viscous. The germinal disc was large (3.5-4 mm in diameter) and in its central region cleavage furrows were seen, sometimes in an excentric position (figs. 31 and 33). As the rate of divisions was very high, five to six divisions during the first 2 hr (from the time of the first cleavage furrow to stage II), we do not refer to each division as a separate stage. We therefore included in stage I the whole range of cleaving patterns in which all cells were still open peripherally, or even those with one to two centrally located and laterally closed cells. Additional stage I germinal discs are shown to demonstrate the variability of cleavage patterns from a very orderly one (Figs. 35 and 36) to another (Figs. 32 and 34) with a single circumscribed cell, indicating asynchrony of the mitotic divisions. The cytoplasm at this stage contains many large vacuoles (vac.). In some of the embryos of this stage a polar body (p.b.) could still be discerned (Figs. 1 and 31). Another relatively common feature is the presence of several knobs (su. sp.) near the periphery, some being minute (the size of a polar body) and others are much larger (the size of a blastomere). The minute knobs resemble the supernumerary sperms of the pigeon (Patterson, 1910), while the larger ones might be nests formed by dividing supernumerary sperms before their disintegration. In a stage I germ observed from the lower side, all the cells seem to be open from below.

Stage II (about 2 hr uterine age; Figs. 3 and 4). On the upper surface there is a group of 14-16 laterally closed cells enclosed by vertical cleavage furrows. The blastomeres are elevated in relation to the originally flat surface of the germ and resemble a nest of eggs. From the central group, cleavage furrows spread out in all directions. The uncleaved cytoplasm still contains vacuoles; however, these are smaller than stage I vacuoles. The lower surface does not yet reveal the formation of horizontal cleavage furrows.

Stage III (3-4 hr uterine age; Figs. 5 and 6). The germ at this stage has a notably smaller diameter than in stage I. This accentuates the tendency, already visible in many stage II germs, that during cleavage the cytoplasmic disc shrinks horizontally and thickens vertically. On the upper surface a central group of 80-90 laterally


Stage I
Stage II
Stage III
Upper Surface
Lower Surface
1
2
3
4
5
6
c.fur
su.sp
p.b
vac
cl.bl
1mm

FIGS. 1–6.

closed blastomeres is seen. From its margin cleavage furrows radiate to the edge of the germinal disc.

On the lower surface, some 10-16 cells (cl. bl.) that are closed ventrally by horizontal furrows can be seen. The more laterally situated vertical furrows of the dorsal side are not deep enough to show on the vertical surface. In the uncleaved cytoplasmic region of the ventral side many vacuoles can still be seen, although they are much smaller than those of earlier stages.

Stage IV (5 hr uterine age; Figs. 7 and 8). The diameter of the germ is similar to that at stage III. On the upper surface there are 250-300 closed cells while in the center of the lower surface 80-90 ventrally closed cells may be seen. The ventral area covered by the closed cells indicates the dimensions of the subgerminal cavity (s.c.) at this stage.

Stage V (8-9 hr uterine age; Figs. 9 and 10). Cleavage is much more advanced. The closed blastomeres occupy equally large areas both on the upper and lower surfaces. The sub-blastodermic cavity has increased remarkably and stretched towards the periphery. However, both surfaces do not yet form smooth areas and appear to be composed of rounded beadlike blastomeres.

Stage VI (10-11 hr uterine age; Figs. 11 and 12). The entire cytoplasmic mass of the germinal disc is cleaved, both on the upper and lower surfaces. The cells are very small and form an epithelial sheet of uniform thickness. The beadlike appearance of the individual cells is lost and the germ can legitimately be called a blastoderm.

Period B: Formation of Area Pellucida (Stages VII-X)

Stage VII (12-14 hr uterine age; Figs. 13 and 14). The cells of the upper surface became much smaller as a result of intensive dividing, although at the magnification used by us it is difficult to distinguish the individual cells. The cells of the lower surface are, however, much larger and generally similar in size to the cells of the lower surface of stages V-VI.

The epitheliumlike appearance of stage VI is modified as a result of the shedding of some cells from a limited area of the lower surface at the posterior half of the germ. The shed cells rest on top of the yolk at the bottom of the subgerminal cavity. The region that shed the lower cells forms a transparent thinned-out area in the posterior half of the germ and is the first sign of the area pellucida (a.p.). From this stage onward the diameter of the germ increases with progressive development.

Stage VIII (15-17 hr uterine age; Figs. 15 and 16). The transparent region has spread to both sides and forms a sicklelike area. It is now obvious that a marginal band of the germ, to become the area opaca (a.o.) is not included in the new process and does not change its appearance. From the ventral side, the still very large beadlike cells of the lower surface (y.l.c.) can be seen to lose contact and fall off.

Stage IX (17-19 hr uterine age; Figs. 17 and 18). The transparent region is now

FIG. 1. Upper surface of stage I germ demonstrating cleavage furrows (c. fur.) polar body (p.b.) nests of supernumerary spermia (su. sp.).

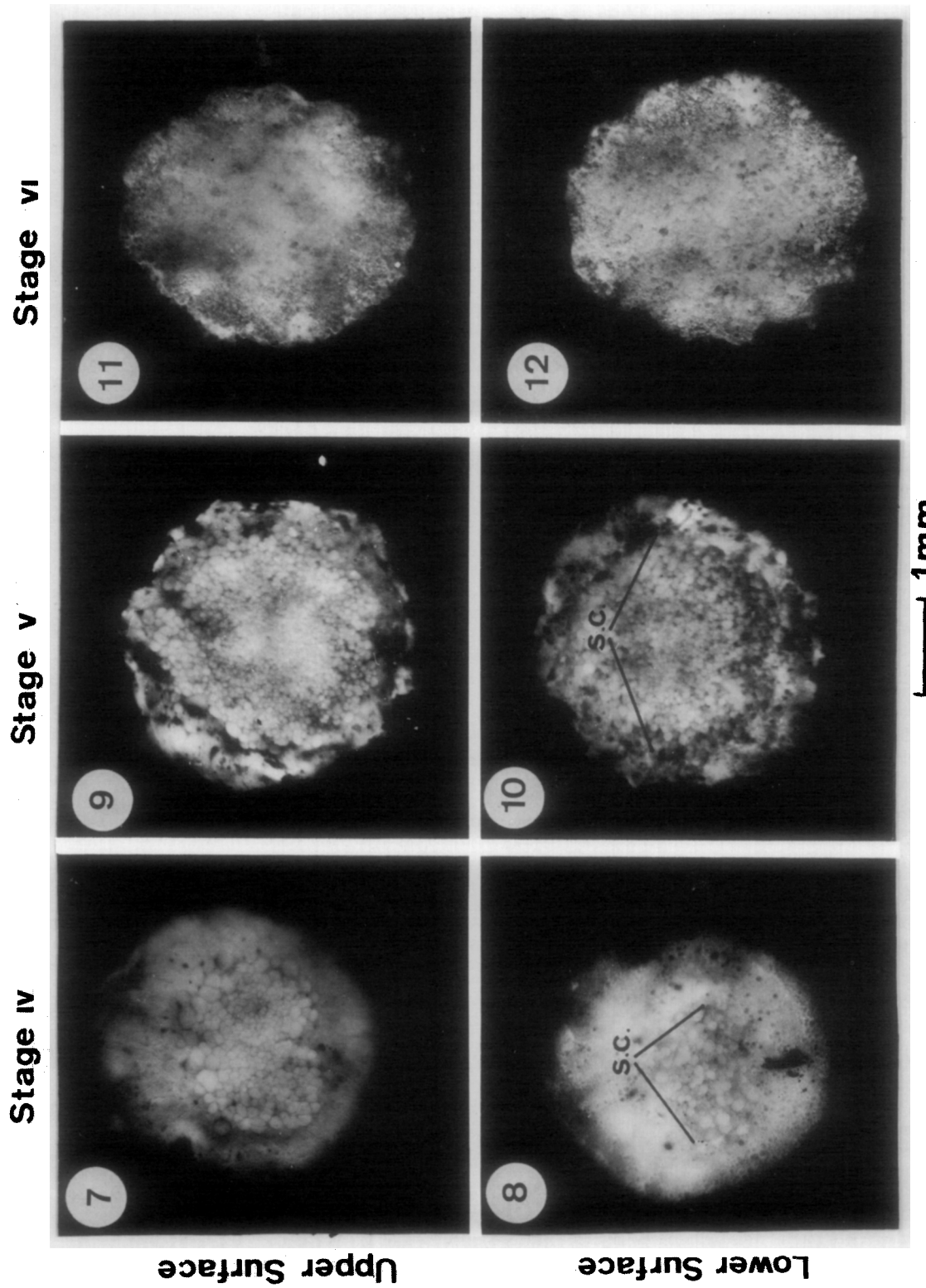
FIG. 2. Lower surface of same stage I germ. No cell boundaries discerned. Large vacuoles (vac.) randomly distributed.

FIG. 3. Upper surface of stage II germ. Cleavage has proceeded. More centrally located ones still open laterally.

FIG. 4. Lower surface of same stage II germ. No change from stage I (Fig. 2) except for small vacuoles.

FIG. 5. Upper surface of stage III germ. Cleavage proceeds towards the periphery. Central blastomeres smaller than more laterally located ones.

FIG. 6. Lower surface of same stage III germ. At the center a few blastomeres are seen closed off also on their ventral side (cl. bl.). Vacuoles numerous, remarkably smaller, occupy area lateral to closed off blastomeres.


Stage IV
Stage V
Stage VI
Upper Surface
Lower Surface
7
8
9
10
11
12
S.C.
S.C.
1mm

FIGS. 7–12.

spreading in an anterior direction, although the process is not yet completed and the border between the area opaca and the area pellucida is not yet a sharp one, especially at the anterior end.

Stage X (a freshly laid egg about 20 hr uterine age; Figs. 19 and 20). Observation both from the upper and lower surfaces reveals that the formation of the area pellucida has been completed and there is a clearly demarcated border between it and the area opaca. However, this stage not only terminates period B (the formation of the area pellucida), but is also the beginning of the following period C. On closely observing the lower surface, clusters of small cells (much smaller than the cells shed during the formation of the area pellucida) are seen to form a meshlike layer at the posterior area of the blastoderm i.ag.). Only the most posterior region of the area pellucida fails to be involved in this new process and remains as a transparent sickle shaped belt (t.b.).

Period C: Hypoblast Formation (Stages XI–XIV)

All the stages included in this period and onward are arrived at by incubating the freshly laid egg for varying lengths of time. Incubation time depends in each case on the initial stage of the embryo, which in turn depends on the time lapse since laying and on the environmental temperature. Although the ventral cell clusters of stage X may be regarded as the first signs of hypoblast formation, from a morphologic point of view, it is preferable to refer to an actual structure, which can be directly related to the hypoblast.

Stage XI (Figs. 21 and 22). Observation of the upper surface of the blastoderm reveals a smooth thin layer through which deeper concentrations of cells may be seen. The same picture, though slightly better defined, may be seen when looking at the ventral side of the blastoderm. Here again in front of the posterior section of the area opaca a transparent beltlike area (t.b.) may be seen, the anterior border of which is demarcated by a relatively narrow horseshoelike concentration of cell clusters (k.s.). Inside this horseshoe a number of individual clusters of various sizes may be seen (i.ag.). The cell clusters demonstrate a clear antero-posterior orientation with the bigger ones at the posterior side and the smaller ones situated more anteriorly. The horseshoelike concentration of cells at the posterior side of the blastoderm is probably what is referred to in the literature as Koller's sickle and from a morphologic point of view, the beginning of the hypoblast.

Stage XII (Figs. 23 and 24). Here again the transparent posterior belt may be seen, anterior to which the newly formed lower layer, the hypoblast, already lines half of the lower surface of the area pellucida. The hypoblast, although, sheetlike, is not entirely continuous and gives the impression of being formed by the fusion of separate cell masses.

Stage XIII (Figs. 25 and 26). The process of hypoblast formation has been completed. The posterior margin of the hypoblast is still very accentuated on the ventral side and also may be seen very clearly from the dorsal side through the transparent epiblast. The upper surface continues

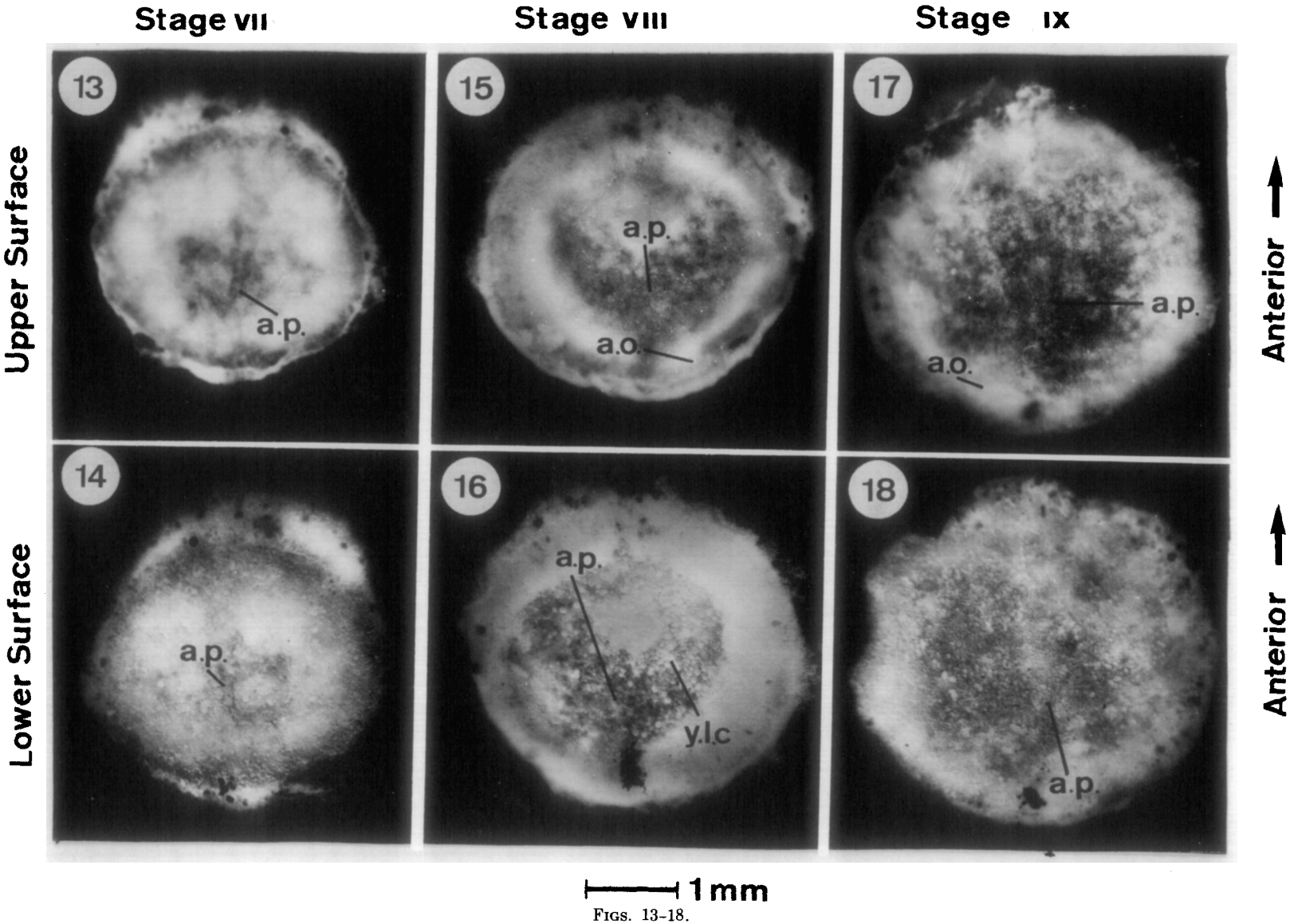
FIG. 7. Upper surface of stage IV germ. Cleavage proceeds. Central cells becoming smaller; lateral cleavage furrows reach margin of germ.

FIG. 8. Lower surface of same stage IV germ. Amount of ventrally closed blastomeres increases. Beneath latter subgerminal cavity (s.c.) is expanding.

FIG. 9. Upper surface of stage V germ.

FIG. 10. Lower surface of same stage V germ. Almost all blastomeres closed ventrally and subgerminal cavity (s.c.) remarkably enlarged.

FIGS. 11, 12. Upper and lower surface views of same stage VI blastoderm. Fully cleaved, individual cells have lost beadlike form and are incorporated into epithelial sheet. Upper and lower surfaces appear similar.


Stage VII
Stage VIII
Stage IX
Upper Surface
Lower Surface
13
15
17
14
16
18
a.p.
a.o.
y.l.c
Anterior
Anterior
1mm

FIGS. 13–18.

to be smooth without any sign of depression or invagination.

Stage XIV (Figs. 27 and 28). The anterior side of the hypoblast now also has well-defined borders and at the posterior side a cellular bridge is being formed between the hypoblast and the area opaca (pos. br.).

Hamburger–Hamilton stage 2 (Figs. 29 and 30). Here the primitive streak (p.s.) can be seen to form in the cellular bridge posterior to the initial posterior margin of the hypoblast.

DISCUSSION

Although much work has been done on early stages of chick development during the last 100 years, there is nevertheless confusion about development prior to primitive streak formation. To date, no systematic study has been made of this entire period, and the existing fragmentary data suffer from drawbacks. The main disadvantage of the sporadic data was that the material had been studied after fixation and generally only from microscopical sections. In those cases in which sectioning was attempted according to Von Baer's law (which is known to be inaccurate), a deviation of a few degrees might have lead to misinterpretations. Also, no criteria existed to arrange the data gathered from all the different observations in a correct temporal developmental order.

The aim of the present study was therefore to rectify the above-mentioned shortcomings by systematically studying one stage after another with an exact timing of the stages; by studying live material, photographing it as promptly as possible, and paying attention to the exact anterio-posterior orientation of the embryo. The present study deals only with the information gained by using a stereoscopic microscope; however, parallel material that was collected and processed for both light and electron microscopy has been studied and will be discussed separately.

The developmental period least affected by the above-mentioned drawbacks is the cleavage period. Here, even without timing, there can be no doubt about the order of the stages. The studies of both Patterson (1910) and Olsen (1942) provide quite accurate correlation between the time that elapsed from the laying of the previous egg and the number of blastomeres seen in surface view. Neither of these authors, however, attempted to stage the germs of the period studied, to photograph them in a live condition, nor to observe the germ's lower surface while checking its relation to a subgerminal cavity. Patterson even mentions that in the hen's egg, photographs of the living cells are impossible, this in contrast to the pigeon's egg in which the cleavage furrows stand out clearly; the early stages of the latter were successfully photographed by Blount (1909). Probably, it was Patterson's statement that discouraged later investigators from attempting to deal with the living germ. Olsen (1942) also fixed the embryo and stained it with gentian violet to accentuate the cleavage furrows. In the present study, it was found that if a living germ is removed from the yolk it can be photographed on a black background when correctly illuminated.

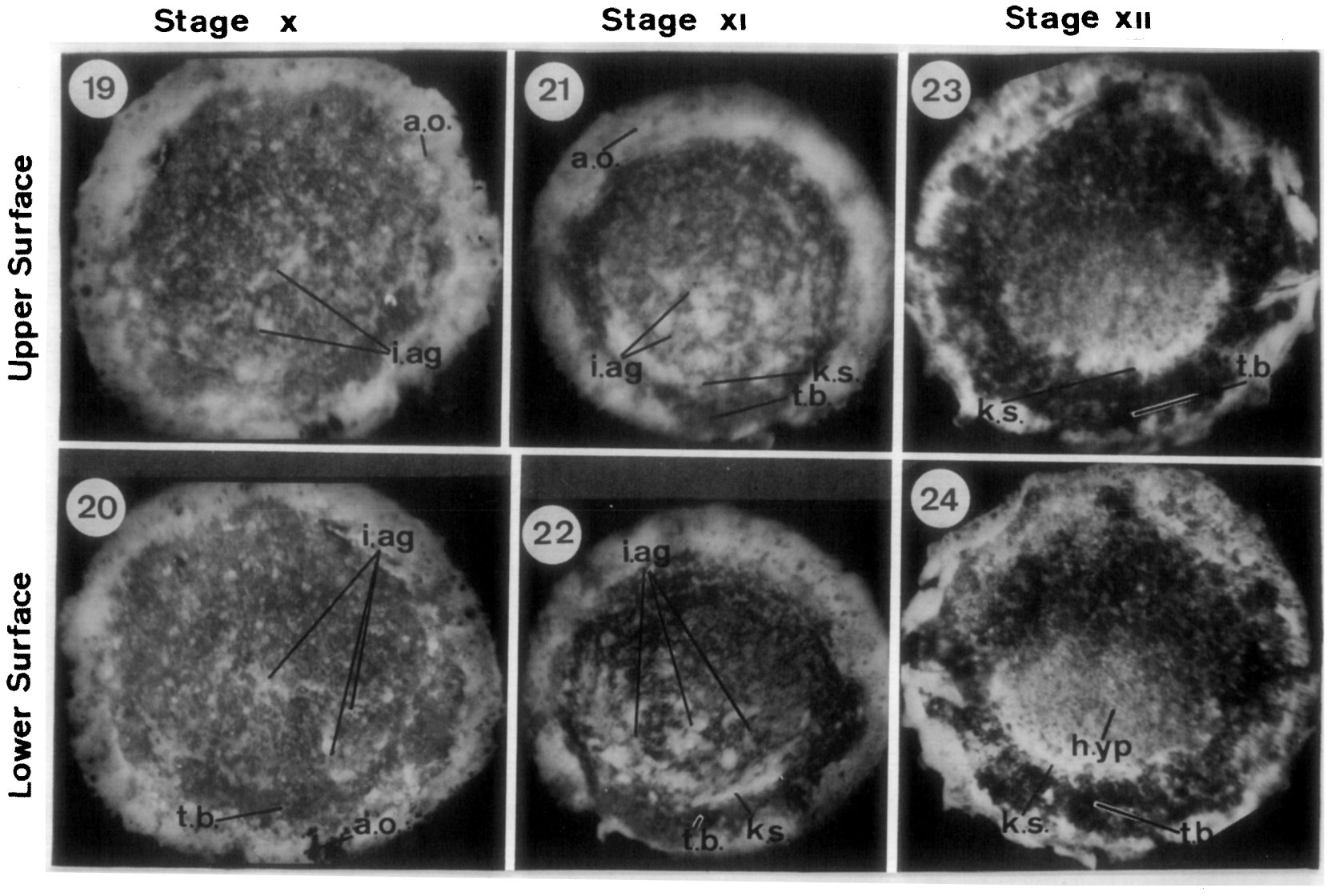
FIG. 13. Upper surface of stage VII blastoderm. Upper surface remains smooth, but its posterior half becoming transparent, indicating formation of area pellucida (a.p.).

FIG. 14. Lower surface of same stage VII blastoderm. Area pellucida formed through process of cell dropping.

FIG. 15. Upper surface of stage VIII blastoderm. Area pellucida expands from original posterior region in anterio-lateral direction. Marginal area, the area opaca (a.o.), remains unaffected by this process.

FIG. 16. Lower surface of same stage VIII blastoderm. Shedding off of ventral yolk laden cells (y.l.c.) is clearly seen.

FIGS. 17, 18. Upper and lower surface of same stage IX blastoderm. Formation of area pellucida nearly completed.

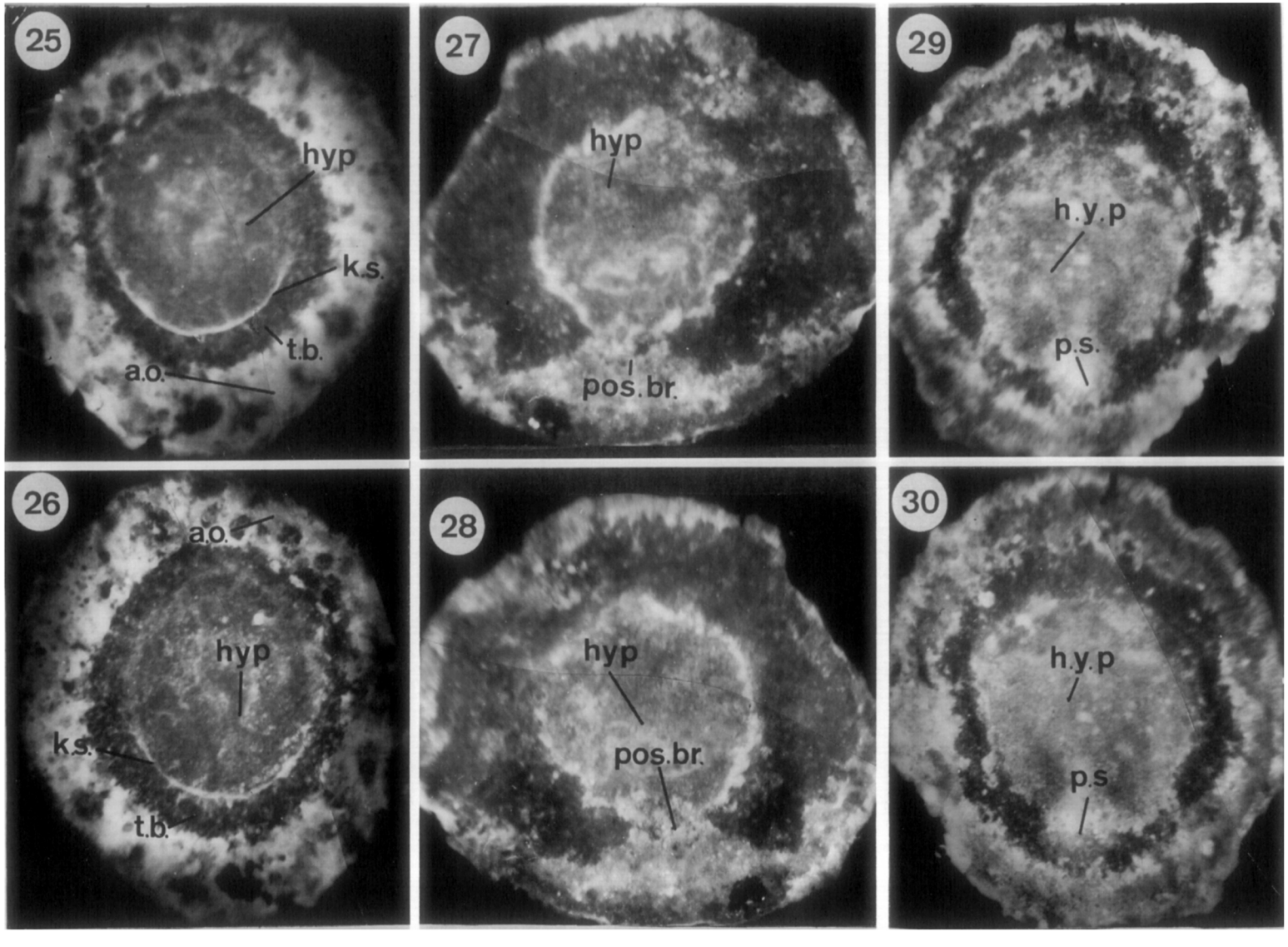

Stage x
Stage xi
Stage xii
Upper Surface
Lower Surface
Anterior
Anterior
19
a.o.
i.ag
21
a.o.
i.ag
k.s.
t.b
23
t.b
k.s.
20
i.ag
t.b.
a.o.
22
i.ag
t.b.
ks
24
h.yp
k.s.
t.b

Not only are the furrows visible, but in certain cases also the polar bodies (p.b.) and the supernumerary spermia (su. sp.), exactly in the same way as in the pigeon (Blount, 1909). By observing the first cleavages in many germs (Figs. 31–36) it may be seen that cleavage can start at any point in the germ, that the first furrow which is often eccentric does not divide the germ into two equal parts, and therefore, has nothing to do with the future axis. This is in accordance with many earlier observations (Duval, 1884; Patterson, 1910; Olsen, 1942). As far as the synchrony of the cleavages is concerned, there is also disagreement among different investigators. A recent report by Emanuelsson (1965) claims that the first five divisions, that is, up to 32 blastomeres, are synchronous, whereas earlier investigators claim that the third cleavage is already irregular (Patterson, 1910; Blount, 1909). Our results agree with the findings of Patterson and Blount, whose observations were based on a much more extensive series of embryos. Generally, it may be stated that in most cases the third cleavage, and sometimes the second cleavage, are already asynchronous and irregular, although occasionally regular cleavages also may be found (Figs. 35 and 36). Another feature that is very prominent in live, cleaving germs is the abundance of vacuoles which are large in the earliest stages and become progressively smaller until they disappear altogether at stage VI, the end of cleavage. Most investigators do not mention the vacuoles, and here again attention was paid to them by workers such as Disse (1878), who disputes with His (1968) about the latter's belief that there is a progressive accumulation of vacuoles that later contribute to the formation of the subgerminal cavity. Also, Goette (1874) and Oellacher (1869) attributed the formation of the subgerminal cavity to the accumulation of fluid. Disse experimented with fixation and concluded that the formation of vacuoles is not physiologic, but an artifact caused by the chromic acid. All the above investigators neglected looking at a nonfixed embryo which readily demonstrates an abundance of vacuoles. Although we cannot yet say anything concerning the connection between the vacuoles and the subgerminal cavity, we can state that such a cavity filled with fluid does exist. Here again, there is in the literature a discussion based on microscopical slides on whether the cavity, seen by many investigators, is real or an artifact. Jacobson (1938) and Pasteels (1945) believe that there is only a narrow slit between the germ and the underlying yolk, the cavity seen in the slides being artifactual. Clavert (1960, 1962), who based his conclusions on observations of live material, but of the intact egg without trying to remove the germ from the underlying yolk, is even more radical. He believes that only 6–8 hr before the egg is laid (our stages VII–VIII) "the germ detaches itself from vitellus . . . the area pellucida then appears, off center." Our material demonstrates that the subgerminal cavity exists from stage III onward. It begins in the region where the first cells close off ventrally, as a very small centrally situated cavity, and it then extends gradually towards the periphery to spread under the entire germ at stage

FIGS. 19, 20. Upper and lower surface of same stage X blastoderm. On lower surface of thinned out blastoderm isolated cell aggregates (i. ag.) appear, more concentrated at posterior half. Transparent belt (t.b.), however, separates aggregates from area opaca (a.o.).

FIGS. 21, 22. Upper and lower surface of same stage XI blastoderm. Most posterior aggregates coalesce to form Koller's sickle, anterior to transparent belt.

FIGS. 23, 24. Upper and lower surface of same stage XII blastoderm. Koller's sickle being its posterior margin, a hypoblast (hyp.) is forming progressing in an anterior direction. At this stage hypoblast underlies only posterior half of the epiblast.


Upper Surface
Lower Surface
25
hyp
k.s.
t.b.
a.o.
27
hyp
pos.br.
29
h.y.p
p.s.
Anterior
26
a.o.
hyp
k.s.
t.b.
28
hyp
pos.br.
30
h.y.p
p.s
Anterior
1 mm

FIGS. 25–30.

VI. This cavity is filled with fluid and can be penetrated easily with a needle during the operation.

This brings us to consider the true meaning of the appearance of the area pellucida (a.p.). The formation of the a.p. was a neglected period in the chick's development probably because it is intrauterine and because it is relatively short and takes 4–6 hr. Most of the investigators did not even realize that this period is a very critical one in development and were concerned only with the existence of the underlying cavity. It was Clavert (1960, 1962) who stressed the connection between the appearance of the a.p. and the attaining of bilateral symmetry by the germ. Clavert realized that he could change the orientation of the embryo by rotation in different directions, but this was effective only "as long as the area pellucida has not begun to appear. The formation of the a.p. corresponds with some important transformation."

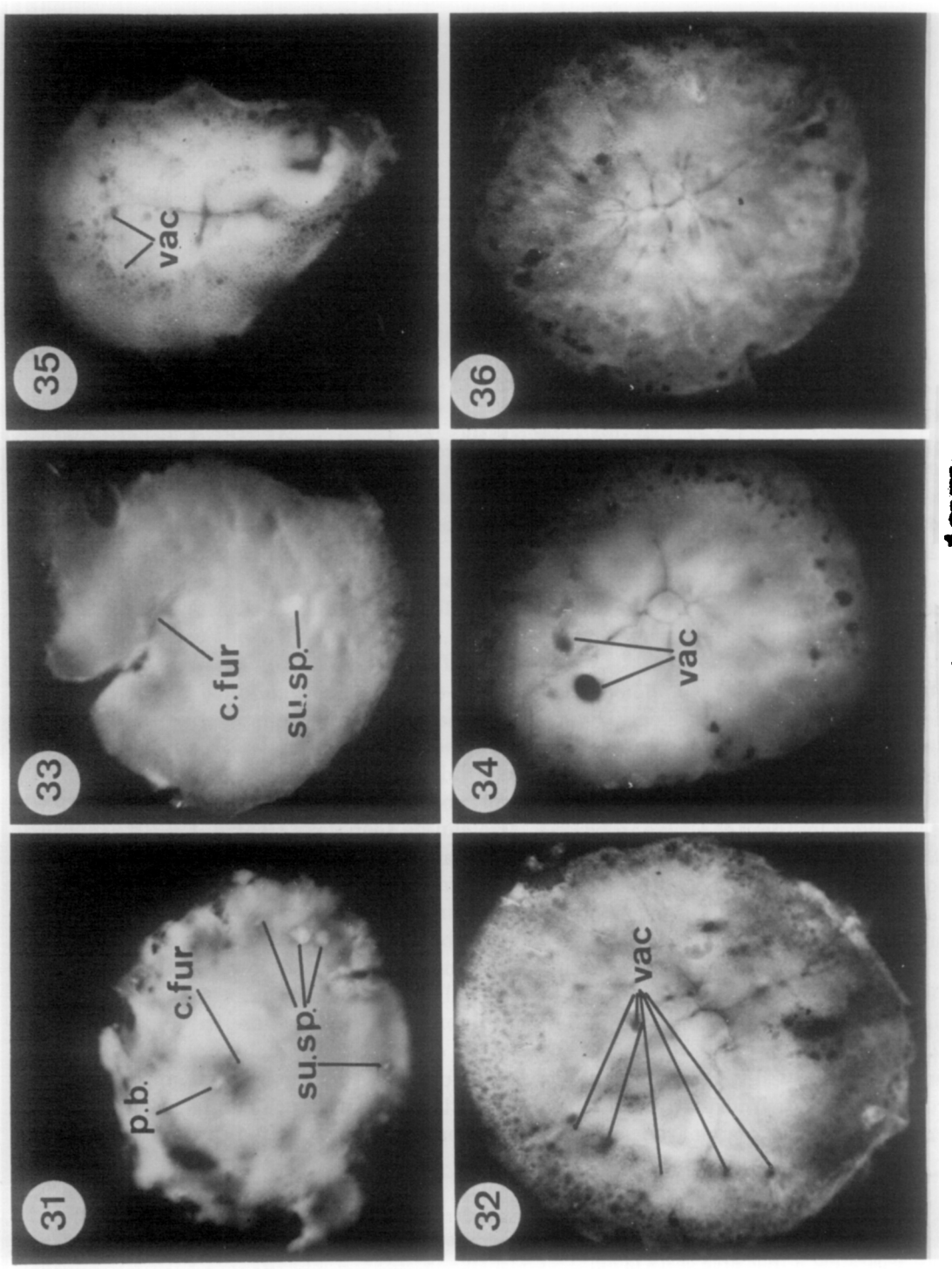
By trying to analyze the phenomenon of symmetrization (Kochav and Eyal-Giladi, 1971) we found that it was not rotation but the force of gravity that induced symmetry in the germ. By hanging the yolks of stage VI uterine eggs by their chalazae in beakers filled with Ringer's solution we were able to observe the process of symmetrization, and we saw that the a.p. began to appear at the future posterior side of the germ by the appearance of a transparent area, which gradually extended in an anterior direction. Up to this point we agree with the descriptions of Patterson (1909) for the pigeon and Clavert for the chick, but from here on our interpretations part. Clavert interpreted the appearance of the transparent spot as a "brutal detachment of the germ from the vitellus," which we have now seen to be incorrect. He also agrees with Kolliker (1875) that at the same time a rearrangement of cells occurs so that the germ that was five to six cells thick in its central region, is by then two to three cells thick. The answer to what is going on during the appearance of the transparent a.p. lies again in the observation of the lower surface of the live germ. From stage VII to stage X something brutal happens to the germ, but not a detachment from the yolk. Instead, many cells detach themselves in an orderly way from the ventral side of the multilayered opaque germ, to transform the germ by a gradual process, progressing from the future posterior to the future anterior side, into a thin transparent germ. The shed cells are found in the subgerminal cavity in the form of yolk balls (Dotter-Kugeln of Goette, 1874; Peter, 1938; or Furchungskugeln of Kolliker, 1875), the existence of which could not be explained adequately until now. We describe here the first morphogenetic happening in chick development during which the direction of the embryonic axis is not only determined, but also can be seen very clearly.

We are dealing with two quite different phenomena which have thus far been confused in the literature. The formation of the subgerminal cavity is connected with the closing off of the lower side of the ventrally open blastomeres. This process therefore starts somewhere in the central region of the germ and spreads gradually centrifugally. At the end of stage VI, the cavity is spread under the entire germ and is a real gap filled with fluid. After the

FIGS. 25, 26. Upper and lower surface of stage XIII blastoderm. Full hypoblast (hyp.) is formed. Koller's sickle (k.s.) is still visible at posterior margin. Transparent belt (t.b.) now surrounding area occupied by the hypoblast separating it from area opaca (a.o.).

FIGS. 27, 28. Upper and lower surface of stage XIV blastoderm. At posterior side of blastoderm cellular bridge (pos. br.) develops connecting hypoblast with area opaca.

FIGS. 29, 30. Upper and lower surface of Hamburger and Hamilton stage 2 blastoderm. Posterior bridge is clearly seen and in it beginning of primitive streak (p.s.).



FIGS. 31-36. Six blastoderms of stage I demonstrating different patterns of early cleavage. c. fur., cleavage furrow; p.b., polar body; vac., vacuoles; su. sp., supernumerary spermia.

formation of the cavity is completed, a morphogenetic process, started by the force of gravity, begins in an oriented posterio-anterior direction. This morphogenetic process is externally expressed by the orderly shedding of the lower layers of cells starting at the future posterior and ending at the future anterior side, leaving a denuded central area as a thin transparent sheet—the area pellucida. At the termination of the above-described process, the symmetrized egg is ready to be laid. In the freshly laid egg, two distinct circular areas can already be distinguished, the peripheral area opaca and the central very thin, essentially single-layered area pellucida. By now the symmetrized germ looks radial again and it is difficult for an inexperienced eye to determine its polarity. However very few investigators have faced this problem at the above stage of development (stage X). To get embryos at stage X one usually has to collect the eggs immediately on being laid, remove the germ from the yolk without delay, and inspect its lower surface. Unincubated eggs that are left for a few hours or more at room temperature, continue to develop slowly and when opened are found to be in a much later stage. As most investigators have the eggs supplied to them, they are received at a relatively late developmental stage, which accounts for the numerous faulty descriptions of an unincubated germ. Bellairs (1971) summarizes the literature on this point by saying that "hens' eggs have generally become two-layered" when the egg is laid.

The question of how the germ becomes two-layered has been dealt with by many investigators, most of whom again base their studies on fixed and sectioned material. The literature on this issue is absolutely fascinating, as almost every possible explanation for the formation of the hypoblast has been adopted by somebody. There is at least a general concensus that the germ was once, while inside the uterus, a single-layered embryo and then became double layered. Balfour (1878) believed that the large loose yolk balls (our yolk-laden cells) found at the bottom of the subgerminal cavity, which he named formative cells after Peremeschko (1868), suddenly begin to undergo a process of metamorphosis; from being spherical and nonnucleated, they become flattened and nucleated. These cells form hypoblast first at the center and later at the circumference. Disse (1878) prefers the idea of a gradual formation of the hypoblast from the periphery towards the center in a concentric fashion. Koller (1882) and Duval (1884), followed by Patterson (1909) for the pigeon, Jacobson (1938) for the chick, and Lutz (1955) for the duck, were very anxious to find a homology between the process of hypoblast formation and gastrulation in amphibians and managed to find each in his own way a blastopore either somewhere in the embryonic disc or its posterior margin.

A quite different approach was initially taken by Oellacher (1869), extensively developed by Peter (1939), and later supported by Pasteels (1945), who after studying the duck blastoderm changed his mind about his earlier observation of the chick (1937). They claim that "in the single layered germ clefts appear, progressively separating a superficial continuous epithelium from deeper cells." This process called by them delamination, results in the formation of a bilaminar embryo.

Chen (1932) interpreted the formation of the primary hypoblast in the duck as a forward growth by proliferation of the thickened posterior germ wall. He, however, mentioned that the entoderm in the unincubated blastoderm is a loose layer of separate cells, and that it becomes filled in, to form a continuous coherent sheet during early incubation.

Many other investigators (His, 1868; Nowack, 1902; Merbach, 1935; Pasteels, 1937; Vakaet, 1962; Spratt and Haas, 1965) have noticed that the primary hypoblast starts as a noncoherent layer, especially in the

future anterior region, and interpreted its appearance as a process of "polyinvagination." Some of the above-mentioned authors added other mechanisms to the polyinvagination. For instance, Merbach (1935) advocated in addition an invagination at the posterior end.

Others, like Nowack (1902), Vakaet (1962), and Spratt and Haas (1965), claim that in addition to polyinvagination there is an anteriorly directed movement of cells of the lower layer from a posterior concentration of cells. Of the last group of authors, Nowack denies the existence of a posteriorly situated concentration of cells (Koller's sickle) from which the initial primary hypoblast grows anteriorly, whereas Vakaet (1962) and Spratt and Haas (1965) agree that a concentration of cells reminiscent of Koller's sickle exists, although there is probably no furrow (Sichelrinne) to account for a real invagination (blastopore), as suggested by Koller (1882) himself.

The material presented here demonstrates very clearly all the stages of formation of the primary hypoblast. At stage X, while the area pellucida is completed, the primary hypoblast is already starting to form as isolated clusters (i.ag.) of cells being more crowded at the posterior side of the germ. However, there is a transparent beltlike, pure epiblastic region (t.b.) separating the above aggregates from the posterior area opaca. At stage XI, there is a notable increase in cell population density at the posterior side forming a clearly demarcated sickle (Koller's sickle, k.s.), anterior to which the as-yet noncoherent cell clusters also seem to grow in size. At stage XII, the sickle becomes coherent with the aggregates of the posterior half to form the posterior region of the primary hypoblast, probably the area called by Koller (1882) the embryonic shield and by Peter (1938) the Entodermhof. Also at this stage the posterior sickle and the transparent belt behind it are well defined. The same is also true for stage XIII, at which the hypoblast has been completed. We are confronted with a postero-anteriorly directed second morphogenetic event, following very closely in time and orientation the formation of the area pellucida. Whereas the formation of the area pellucida is characterized by the scattered appearance (polyinvagination) of isolated cells and cell aggregates on the lower surface of the germ. These aggregates coalesce in an orderly manner to form the gradually growing primary hypoblast sheet.

We do not as yet have sufficient data to decide whether the formation of the hypoblast involves only a coalescence of the isolated aggregates or also an active anteriorly directed movement.

Toward the end of hypoblast formation, a cellular bridge appears connecting the central region of the posterior sickle with the posterior area opaca (Figs. 27 and 28, pos. br.). Inside this bridge, the first rudiment of the primitive streak appears. It is not yet clear how this bridge is formed.

After the complete picture of the gross morphologic events has been clarified it is felt that a revision of some terms currently used for the early stages is necessary. The confusion is best demonstrated in textbooks: Patten (1971) uses the term blastodisc for the cytoplasmic disc before cleavage, whereas a blastoderm is a germ after many divisions. Balinsky (1970) inconsistently uses the terms blastoderm and blastodisc for the same developmental stages. Bellairs (1971) does not use the term blastodisc at all and mentions only the term blastoderm. It is therefore suggested that the term germ be employed for the developing chick embryo. The term blastodisc should be avoided, and if desired a germ from stage VI onwards can be called a blastoderm.

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REFERENCES

BALFOUR, F. M. (1873). The development and growth of the layers of the blastoderm. *Quart. J. Micros. Sci.* **13**, 266–276.

BALINSKY, B. I. (1970). "An Introduction to Embryology." Saunders, New York.

BELLAIRS, R. (1971). "Developmental Processes in Higher Vertebrates." Logos Press.

BEKHTINA, V. G. (1960). Early stages of cleavage in the chick embryo. *Arkiv. Anat. Gistol. Embryol.* **38**, 77–85.

BLOUNT, M. (1909). The early development of the pigeon's egg with especial reference to polyspermy and the origin of the periblast nuclei. *J. Morph.* **20**, 1–64.

CHEN, B. K. (1932). The early development of the duck's egg, with special reference to the origin of the primitive streak. *J. Morph.* **53**, 133–187.

CLAVERT, J. (1960). Determinisme de la symétrie bilatérale chez les oiseaux IV Existence d'une phase critique pour symétrisation de l'oeuf. Son stade. *Arch. Anat. Micros. Morph. Exper.* **49**(4), 345–361.

CLAVERT, J. (1962). Symmetrization of the egg of vertebrates. *Advances in Morphogenesis* **2**, 27–60.

DISSE, J. (1878). Die Entwicklung des mittleren Keimblattes im Hühnerei. *Arch. Mikr. Anat.* **15**, 67–94.

DUVAL, M. (1884). De la formation du blastoderme dans l'oeuf d'oiseau. *Ann. Sci. Nat. Zool. VIe. Série T* **18**, 1–208.

EMANUELSSON, H. (1965). Cell multiplication in the chick blastoderm up to the time of laying. *Exp. Cell Res.* **39**, 386–399.

GOETTE, A. (1874). Die Entwicklung der Keimblätter und des Blutes in Hühnerie. *Arch. Mikr. Anat.* **10**, 145–199.

HAMBURGER, V., and HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HARPER, E. H. (1904). The fertilization and early development of the pigeon's egg. *Amer. J. Anat.* **3**, 349–386.

HIS, W. (1868). Untersuchungen über die erste Anlage des Wirbeltierleibes. Leipzig.

JACOBSON, W. (1938). The early development of the avian embryo. *J. Morph.* **62**(3), 415–443.

KIONKA, H. (1894). "Die Furchung Hühnereies." pp. 395–445. Anatomische Hefte I Abteilung X Heft.

KOCHAV, SH., and EYAL-GILADI, H. (1971). Bilateral symmetry in chick embryo determination by gravity. *Science* **171**, 1027–1029.

KOLLER, C. (1882). Untersuchungen über die Blätterbildung im Hühnerkeim. *Arch. Mikr. Anat.* **20**, 174–211.

KÖLLIKER, A. (1875). "Zur Entwicklung der Keimblätter im Hühnereie," Vol. 8, pp. 209–215. Verhandlungen der Physikal.-medicin. Gesellschaft Wurzburg.

LUTZ, H. (1955). Contribution expérimentale à l'étude de la formation de l'endoblaste chez les Oiseaux. *J. Embryol. Exp. Morph.* **3**(1), 59–76.

MERBACH, H. (1935). Beobachtungen an der Keimscheibe des hühnchens vor dem Erscheinen des Primitivstreifens. *Z. Anat. Entw. Gesch.* **104**, 635–652.

NOWACK, K. (1902). Neue Untersuchungen über die Bildung der beiden primären Keimblätter und die Entstehung des Primitivstreifens beim Hünerembryo. "Inaugural-Dissertation." Druk von E. Ebering, Berlin.

OELLACHER, J. (1869). Untersuchungen über die Furchung und Blätterbildung im Hühnereie. *Stud. Inst. Exp. Path. (Wien)* **1**, 54–73.

OLSEN, M. W. (1942). Maturation, fertilization and early cleavage in the hen's egg. *J. Morph.* **70**, 513–533.

OLSEN, M. W. (1932). Orientation of the hen's egg in the uterus and during laying. *Poultry Sci.* **11**, 266–271.

PASTEELS, J. (1945). On the formation of the primary entoderm of the duck (Anas domestica) and on the significance of the bilaminar embryo in birds. *Anat. Rec.* **93**, 5–21.

PATTERN, B. M. (1971). "Early Embryology of the Chick," 5th ed. McGraw-Hill, New York.

PATTERSON, J. TH. (1909). Gastrulation in the pigeon's egg—a morphological and experimental study. *J. Morph.* **20**(1), 65–123.

PATTERSON, J. TH. (1910). Studies on the early development of the hen's egg. I. History of the early cleavage and of the accessory cleavage. *J. Morphol.* **21**(1), 101–134.

PETER, K. (1938). Untersuchungen über die Entwicklung des Dotterentoderms. 1. Die Entwicklung des entoderms beim Hünchen. *Z. Mikr. Anat. Forsch.* **43**, 362–415.

PEREMESCHKO (1868). Über die Bildung der Keimblätte im Hühnerei. *Akadem. Wissensch. Wien, Math. Naturwiss. Abt.* **57**, 499–516.

SPRATT, N. T. (1960). Morphogenetic movements in the lower surface of the unincubated and early chick blastoderm. *J. Exp. Zool.* **144**, 139–157.

SPRATT, N. T., and HAAS, H. (1961). Integrative mechanisms in development of the early chick blastoderm. II. Role of morphogenetic movements and regenerative growth in synthetic and topographically disarranged blastoderms. *J. Exp. Zool.* **147**(1), 57–94.

SPRATT, N. T., and HAAS, H. (1965). Germ layer formation and the role of the primitive streak in the chick. *J. Exp. Zool.* **158**, 9–38.

VAKAET, L. (1962). Some new data concerning the formation of the definitive endoblast in the chick embryo. *J. Embryol. Exp. Morph.* **10**(1), 38–57.