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The roles of node regression and elongation of the *area pellucida* in the formation of somites in avian embryos

By CLAUDIO D. STERN[1] AND RUTH BELLAIRS
Department of Anatomy and Embryology, University College London, Gower Street, London WC1E 6BT, U.K.

SUMMARY

Experiments have been carried out on explanted chick embryos to test certain widely accepted concepts about the role of Hensen's node in somite formation. The relationship between elongation of the *area pellucida* and regression of Hensen's node has also been investigated.

We conclude from these experiments that:

(a) The timing of somite formation is not controlled by the regression of Hensen's node, nor by the shearing of the mesoderm into right and left halves.

(b) Somite size and shape are probably controlled by local conditions in the chick embryo.

(c) Elongation and regression are two different events.

(d) The position of the somites probably depends on mechanical tensions in the *area pellucida*.

(e) The notochord is not required for the stability of somites *in vivo*.

INTRODUCTION

It has often been suggested that Hensen's node plays an important role in the development of somites in the chick embryo. Formerly, it was thought that it acted as an 'inductor' and so programmed the mesoderm to become somites, but this idea became less acceptable when it was shown that somites could form in the complete absence of both the node and its derivative, the notochord (Bellairs, 1963). Indeed, there is now evidence (Bellairs & Veini, 1984) to suggest that determination of at least some of the future somitic cells begins even before the node itself has fully developed.

Another fruitful idea was put forward by Lipton & Jacobson (1974*a*,*b*) who suggested that as it regressed down the primitive streak, Hensen's node mechanically sheared the presumptive somite mesoderm into right and left sides. They suggested that a 'pre-pattern' was present in the primitive streak and that this became stimulated to form somites by the node as it regressed along the streak.

[1]*Present address:* Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, to whom correspondence should be addressed.

Evidence in support of a pre-pattern is discussed elsewhere (Bellairs, 1984), but the possible role of the node in eliciting the segmentation of somites from this pre-pattern will be considered in this paper.

The main evidence presented by Lipton & Jacobson was based on an experiment in which they first removed the anterior part of the *area pellucida*, including Hensen's node, and then divided the posterior part into right and left sides by cutting longitudinally down the primitive streak (Fig. 1B) through the entire thickness of the embryo. They considered that this cutting simulated the action of the regressing node in shearing through the mesoderm. According to these authors, 'during the development of these split fragments, the somites all appeared simultaneously instead of arising in typical anteroposterior sequence'.

In view of the importance of these findings, which have been extensively quoted (e.g. Packard & Jacobson, 1976; Bellairs, 1980; Meier & Jacobson, 1982), we decided to repeat and extend their experiments to determine more precisely the role of regression, as well as of the less studied role of elongation of the *area pellucida*, on the formation of somites.

By *regression* we mean in this context, the anteroposterior movement of Hensen's node along the primitive streak, which results in the laying down of a rod of notochord and which coincides with the gradual disappearance of the primitive streak. By *elongation* of the *area pellucida*, we mean the increase in length which takes place in an anteroposterior direction and which is usually accompanied by a relative narrowing in the mediolateral direction.

During the course of this investigation we found that certain anomalies of segmentation were frequently associated with the use of the agar/albumen culture technique. The study of these anomalies contributed some interesting additional information about the factors involved in the control of somite formation.

MATERIALS AND METHODS

Culture methods

Hen's eggs ('Ross Brown') were incubated up to Hamburger & Hamilton (1951) stages 4–6 in a 38 °C rotating incubator. The embryos were then removed from the yolk and vitelline membrane in Howard Ringer, Pannett-Compton or Tyrodes' saline, and cultured either by a modification (see DeHaan, 1967) of the technique of Spratt (1947) on agar/albumen substrates, or by the technique of New (1955) on the vitelline membranes, or by a combination of the two techniques.

I. Embryos cultured on agar/albumen substrates

The explanted embryos were transferred to 35 mm Falcon dishes each containing a layer of Agar-Glucose-Saline-Albumen (DeHaan, 1967) about 2 mm deep (agar type IV, Sigma Cat. A-7002, Batch 16c-0355). The agar mixture was melted without autoclaving, by briefly boiling over a Bunsen flame.

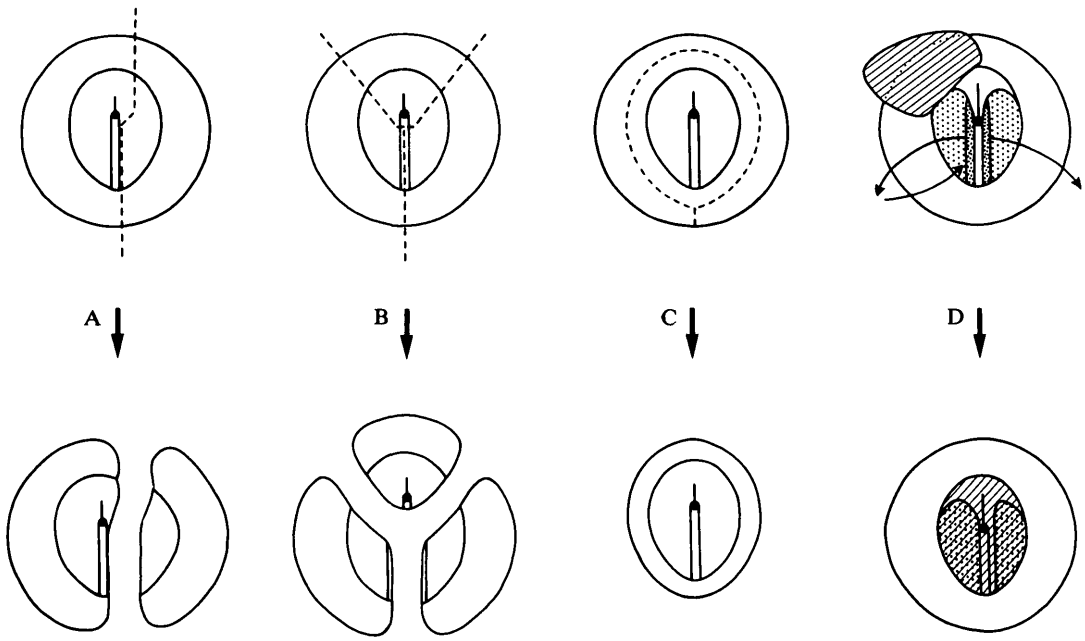


Fig. 1. Schematic diagrams of some of the experiments performed. The upper row of diagrams shows the operations performed, the lower row the resulting embryos or fragments cultured. A; transection of stage-5 embryo paraxial to the primitive streak, equivalent to Lipton & Jacobson's (1974*b*) figure 2. B; transection of stage-5 embryo through the centre of the primitive streak axis, equivalent to Lipton & Jacobson's (1974*b*) figure 8. C; excision of the edge of the *area opaca* in New (1955) culture. D; removal of a strip of paraxial mesoderm. Right: experimental side; the strip is removed and discarded. Left: control side; the strip is removed and replaced (in other embryos this side was left unoperated). After the operation the endodermal layer is replaced.

Seventy-seven embryos were cultured unoperated, dorsal or ventral side down on agar/albumen. Nine embryos were cut into unequal fragments about the primitive streak as shown in Fig. 1A, corresponding to the experiment performed by Lipton & Jacobson (1974*b*, their fig. 2).

Another fifty-two embryos were cut as shown in Fig. 1B, corresponding to the operation described by Lipton & Jacobson (1974*b*, their fig. 8), into three pieces: the first cut ran along the centre of the groove of the primitive streak, and the second and third cuts ran from about 300 μm posterior to Hensen's node to the edge of the *area opaca*, at an angle of about 60° to each other. The cuts were made with tungsten needles sharpened in molten sodium nitrite or with sharp steel iridectomy knives. Any remaining saline in the dishes was then removed with a pipette while arranging the pieces on the surface of the agar/albumen. After the operation, the lids of the Falcon dishes were smeared with thin egg albumen to prevent condensation and dehydration and sealed onto the dishes, which were then incubated at 38 °C.

Other embryos were cultured unoperated, epiblast side down, using a different batch of agar (Sigma batch No. 91F-0367) (ten embryos) or agarose (B.D.H. Batch No. 5518290A) (ten embryos) prepared in the same way as that described above.

II. Embryos cultured by the New (1955) technique

Twenty-six embryos were cultured, each attached to its own vitelline membrane which was stretched around a glass ring over a pool of albumen. The technique originally described by New (1955) was modified in that the embryos were cultured in 35 mm plastic culture dishes (Falcon) instead of in watch glasses.

Ten embryos were operated as follows: about half the width of the *area opaca* was trimmed away around its entire circumference (Fig. 1C). Each embryo was then cultured on its vitelline membrane, and measurements were made every 2–5 h of the length of the *area pellucida*, the distance between the caudal end of Hensen's node and the caudal edge of somite 2, and the outer diameter (measured along the same axis as the embryo) of the *area opaca* remaining around the embryo.

Another eight embryos were operated as follows in New (1955) culture (Fig. 1D): the endodermal layer was carefully lifted on both sides of the primitive streak along its entire length at Hamburger & Hamilton stage 5. A thin (~100 μm) strip of paraxial mesoderm was removed from the area between the posterior end of the primitive streak and as far as possible anterior to Hensen's node. On one side of the embryo the excised mesoderm was discarded. On the other side it was replaced to serve as a control in four embryos, whilst in the remaining four embryos the mesoderm in the control side was left unoperated.

III. Embryos cultured by a combination of the New and agar/albumen techniques

Seven embryos were explanted, each attached to its own vitelline membrane according to the technique of New (1955) and then transferred (vitelline membrane downwards) onto an agar/albumen substrate prepared as described above, so that it was separated from the substrate by its vitelline membrane.

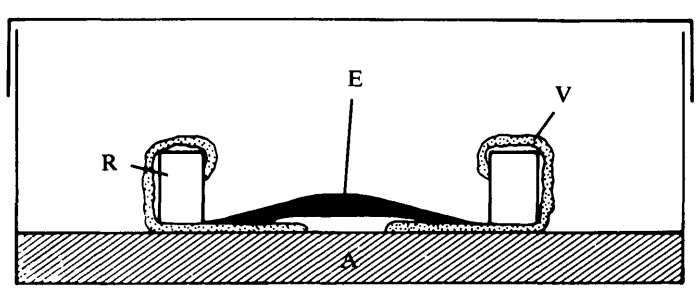


Fig. 2. Schematic diagram of the experimental setup used for the culture of embryos on their own vitelline membranes on agar/albumen. The diagram shows the embryo (E) attached to its vitelline membrane (V) in which a hole has been made to allow contact between the embryo and the agar/albumen. A, agar/albumen gelled in 35 mm culture dish; R, glass ring.

Another twenty-eight embryos were also grown on agar/albumen on their vitelline membranes but a 0·5–1·5 mm diameter hole was cut in each vitelline membrane just under the *area pellucida* to allow direct contact between the embryo and the surface of the agar/albumen (Fig. 2) whilst preserving the attachment of the edge of the *area opaca* to the vitelline membrane.

Filming and analysis of results

Time-lapse filming was recorded on Ilford PanF 16 mm film, using a Bolex H16 camera mounted onto a Zeiss Standard WL microscope placed in a chamber maintained at 37–38 °C by a Sage air-curtain incubator.

The development of control and experimental embryos was followed either by time-lapse filming using a ×1 objective and a frame interval of 1 min, or by taking 35 mm photographs at 2–5 h intervals over a period of about 36 h. Embryos which produced somites visible in the living preparations were fixed in buffered formal saline, embedded in paraffin wax, transversely sectioned at 8 μm, stained with Harris' haematoxylin and mounted in Canada Balsam.

The rate of elongation of the *area pellucida* was calculated from the rate of change of the total length of the *area pellucida*. The rate of regression of Hensen's node was estimated from measurements of the distance between the posterior edge of somite 2 and the primitive pit. These values were measured either from living specimens at 2–5 h intervals using an eyepiece micrometer on a dissecting microscope, or every 60 frames (equivalent to 62 min) from time-lapse films.

The change in width of the *area pellucida* was also determined in some embryos. These were measured just posterior to Hensen's node at stage 5–6, this position was marked by placing carmine particles on the blastoderm, and subsequent measurements were made at the same location.

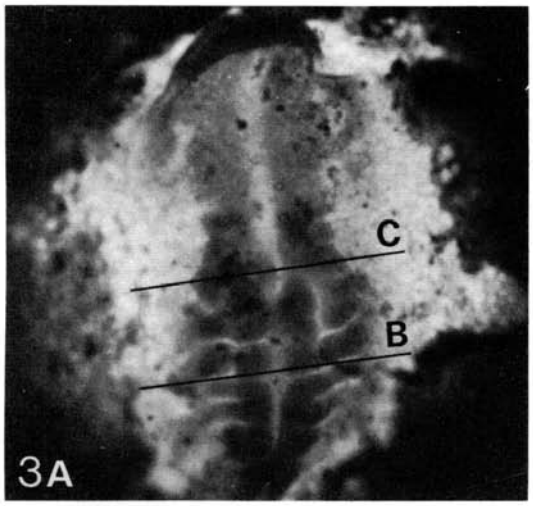
RESULTS

Transection into unequal portions about the primitive streak

Nine embryos were operated as shown in Fig. 1A, to repeat the experiment originally performed by Lipton & Jacobson (1974*b*, their fig. 2). Similar results to theirs were obtained, the only apparent difference being that in our experiments the fragment containing the primitive streak often developed a double row of somites. As in their experiments, any somites which formed on the side which did not contain the primitive streak tended to disperse with prolonged culture. Eight out of the nine embryos showed the above results, and the remaining one failed to develop.

Transection into equal portions about the primitive streak

Fifty-two embryos were operated as shown in Fig. 1B (originally described by Lipton & Jacobson, 1974, their fig. 8) and cultured either dorsal or ventral side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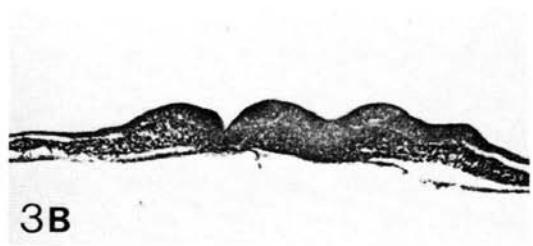
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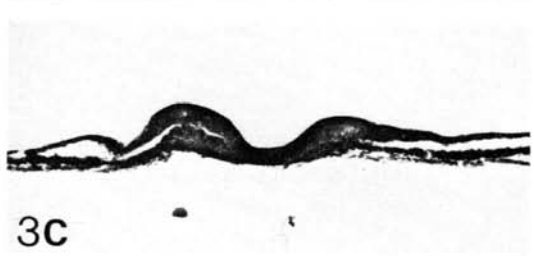
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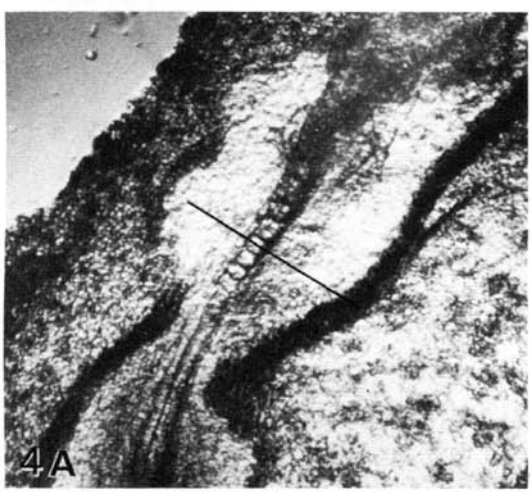
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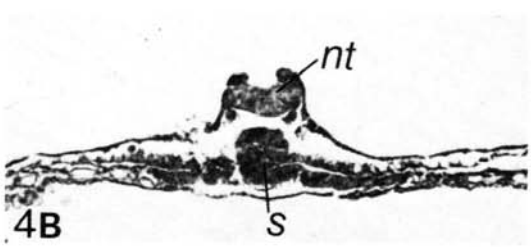
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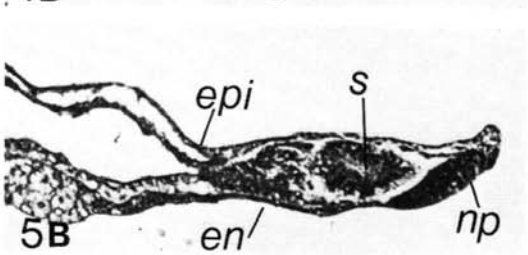
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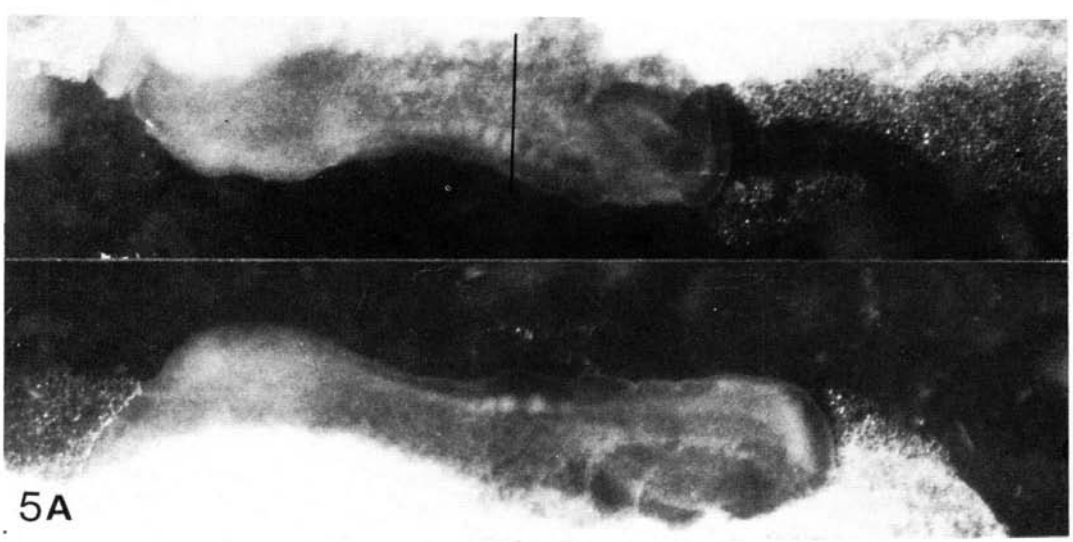
5A

down. Sixteen of these were followed by time-lapse filming and the rest by photographing at intervals of 2–5 h over a 36 h period. As in Lipton & Jacobson's experiments, our experimental embryos produced somites in each of the lateral halves (Fig. 5). Unlike the fragments in their experiments, however, none of our lateral fragments ever made somites simultaneously along their length, but always made them in an orderly cephalic-to-caudal progression, as in normal development (Fig. 6). The time-lapse films also showed that the segmental plate mesoderm separated from the cut edge in an orderly craniocaudal sequence. Transverse sections showed (Fig. 5B) that the structures identified as somites in the living embryos and whole mounts had the cell arrangement characteristic of somites. The sections also confirm Lipton & Jacobson's observations that a portion of the neural plate is contained in each lateral fragment, and that the notochord is absent (Fig. 5B). The anterior fragment usually contained the notochord and a row of somites on either side of it, a head fold, foregut, and the heart. Heart rudiments were also present in the lateral fragments and extra-embryonic blood islands were found in all three fragments. The results were similar whether the fragments were cultured dorsal or ventral side against the agar/albumen.

Removal of a strip of paraxial mesoderm

Eight embryos were explanted in New (1955) culture and operated by removing a strip of mesoderm from one side of a stage-5 embryo as described in the Methods, and four of them were then followed by time-lapse filming. The opposite side of the embryo either remained unoperated (n = 4) or had a similar strip removed and replaced (n = 4), and served as control. The operated embryos made somites in the normal manner and were undistinguishable from unoperated embryos in the pattern and timing of developmental events. Somite segmentation in the experimental side always took place in synchrony with the control side.

Development of unoperated embryos cultured on agar/albumen

Of the seventy embryos cultured with the *epiblast surface* against the agar/albumen, forty-eight (68·6 %) segmented their mesoderm into the abnormal

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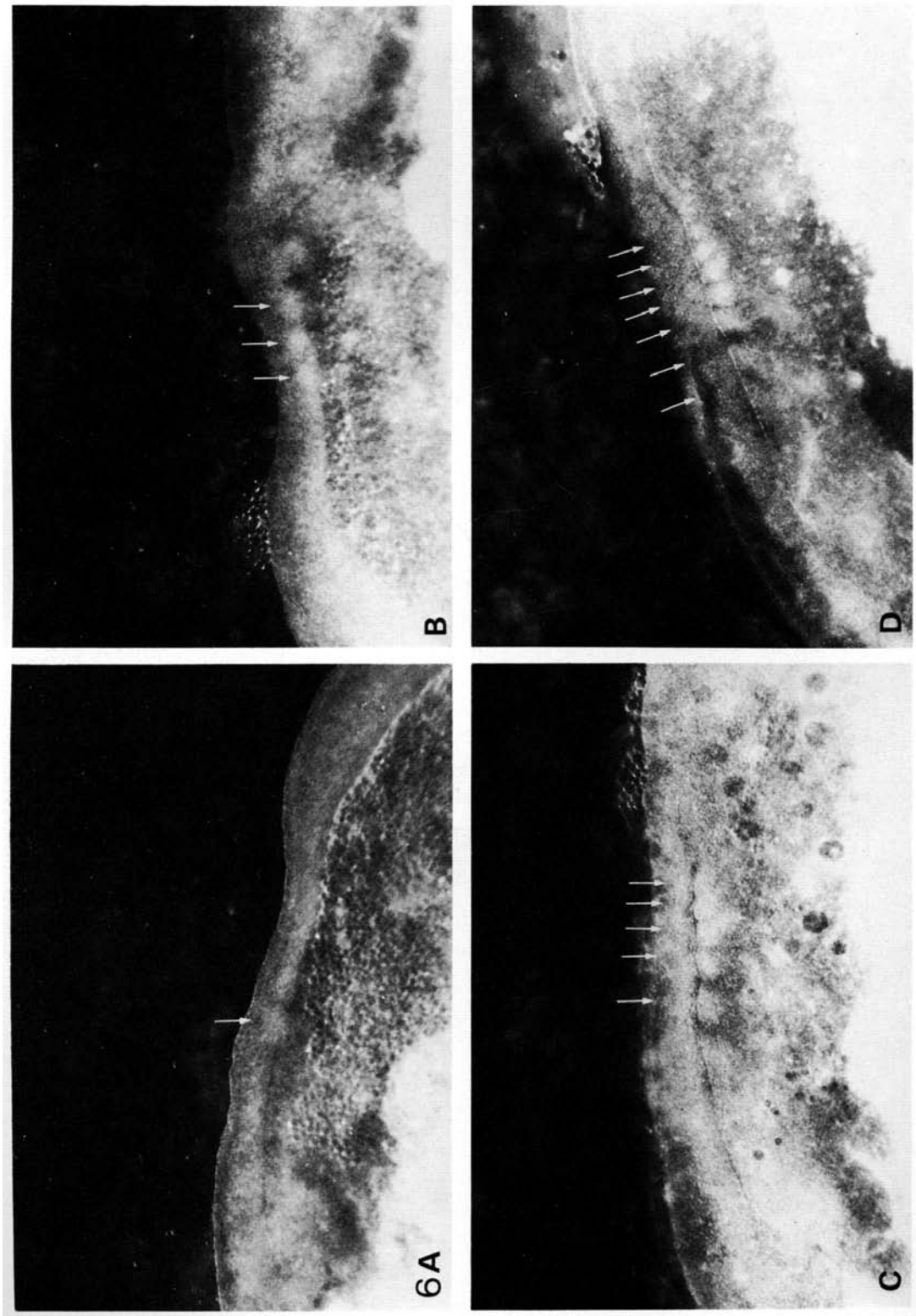
Fig. 3. Folds resulting in an embryo cultured with its ventral surface against the agar/albumen substrate. (A) transmitted light photograph of the living embryo (×15). (B), (C) sections at the levels shown in Fig. 3A (×45).

Fig. 4. Spontaneous case of notochordless embryo. (A) photograph of whole mount of quail embryo after fixation, staining with alcoholic cochineal and mounting in oil of cedarwood (×18). (B) section through the same embryo showing the absence of a notochord and the presence of a single, medial somite (*s*). *nt*: neural tube. (×60).

Fig. 5. Typical result of experiment shown in Fig. 1B. (A) reflected light photograph of a living embryo after 9 h culture (both left and right halves are shown) (×20). (B) section through the embryo shown in Fig. 5A, at the level indicated by the line (×120). *s*: somite; *np*: neural plate; *epi*: epiblast; *en*: endoderm.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patterns shown in Figs 7–9. The pattern of wide (about 1 mm) 'rods' of mesoderm extending laterally from the midline (Fig. 7) was seen by about 8 h culture after explanting ('Pattern (a)'). In contrast, the disturbed arrangement of somites shown in Figs 8 and 9 ('Pattern (b)') was seen only in embryos which were cultured beyond 10–12 h. The somites in these embryos were often arranged as 'bunches of grapes' (Fig. 8), with the greatest number nearest the midline and decreasing in number towards the lateral edges of the embryo. Both patterns could coexist in different regions of a single embryo (Fig. 9). The maximum number of rods observed in pattern (a) was three, and the maximum number of rows of somites seen on each side of pattern (b) was five. Both patterns were found with various degrees of severity. Pattern (a) could merely consist of a single wide 'rod' (Fig. 7), and similarly, pattern (b) could consist of laterally duplicated somites 1 and 2 (Fig. 9).

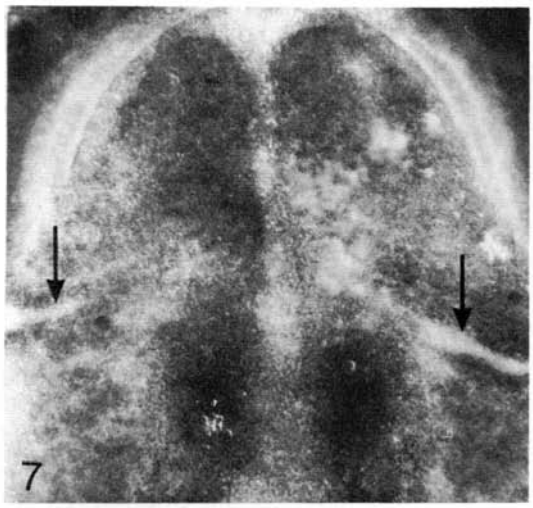
The formation of the abnormal pattern of somites shown in Fig. 8 was seen to occur either as a result of the segmentation of the wide rods (Figs 7 & 9) (26/33 cases recorded), or by direct segmentation of the mesoderm into this pattern (3/33 cases). The somites were of normal appearance in both whole mounts (Figs 8A, B and 9A, B) and sections (Figs 8C, 9C).

One naturally occurring twin embryo (complete duplicitas, primitive streaks side-by-side) was also cultured, epiblast side down. Both sides of this embryo made pattern (a) initially, followed by pattern (b), but they were not synchronous in the formation of either of these patterns. The number of somites was different in the two axes.

Embryos cultured on agar/albumen with their *ventral surface* against the substrate (n = 7) often wrinkled soon after explanting (Fig. 3). The most severely affected embryos also developed the abnormal patterns of segmentation described above.

The abnormal patterns of segmentation were also seen in embryos cultured using a different batch of agar as well as in those cultured on agarose/albumen (see Methods). In order to test whether direct contact between the embryo and the surface of the agar is required for the abnormal patterns of segmentation to occur, two experiments were designed: (a) Embryos were each cultured with their vitelline membrane intervening between the epiblast and the agar/albumen. These embryos developed normally (n = 7). (b) Embryos were each cultured on their vitelline membrane on agar/albumen, but direct contact between the epiblast and the surface of the agar/albumen was allowed through a hole cut in the vitelline membrane (Fig. 2). 17/28 embryos (60·7 %) developed abnormal patterns of somites like those described. Ten out of the eleven embryos

Fig. 6. Frames from a time-lapse film of a fragment of embryo operated as shown in Fig. 1B. (A) 6 h; (B) 8·5 h; (C) 12 h; (D) 15 h. (×35). The vertical lines show the position of the forming somites. Note that the somites form in orderly progression, not simultaneously.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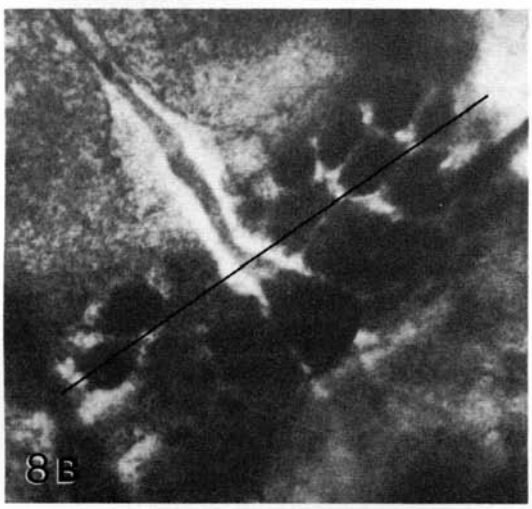
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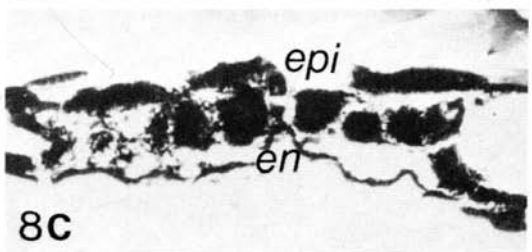
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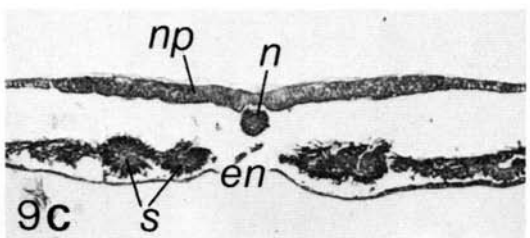
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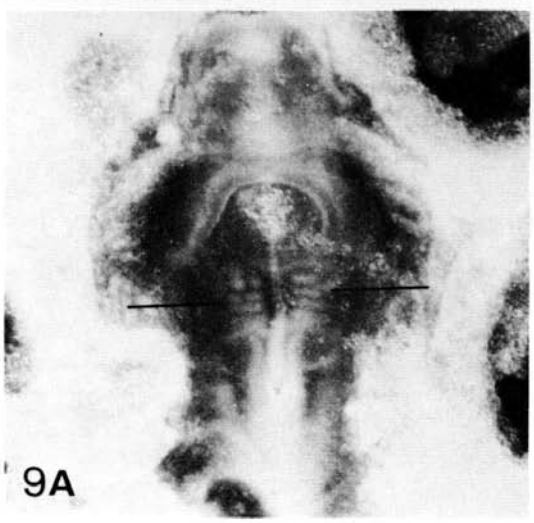
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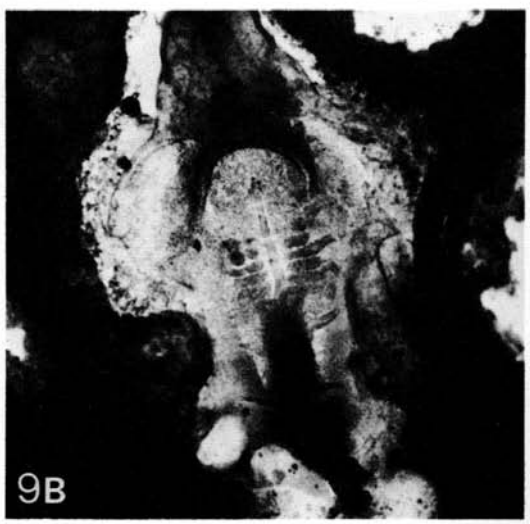
9B

which did not produce abnormal patterns of somites were found not to be attached firmly to the surface of the agar/albumen, but separated from it by a thin layer of fluid. Two of the embryos which had originally been cultured over a hole in the vitelline membrane displaced themselves from this region onto an area of intact vitelline membrane and did not segment abnormally.

The somites tended to disperse after prolonged culture as in the notochordless pieces described by Lipton & Jacobson (1974*b*) and Bellairs & Veini (1984). This phenomenon, however, took place even in the most proximal row of somites in those embryos which possessed multiple rows. Dispersion appeared to take place in a lateral-to-medial direction, with the somites nearest the notochord–neural tube axis dispersing last.

Excision of area opaca edge in New (1955) culture

Embryos (n = 10) which were cultured by the New (1955) technique, each on its vitelline membrane after about half the width of the *area opaca* was removed (Fig. 1C) displayed reduced rates of regression and of *area pellucida* elongation (see below and Figs 10, 12). In spite of the inhibition of elongation, the somites which formed were normal in appearance.

Rates of regression and elongation

The rates of elongation of the *area pellucida* and of regression of Hensen's node were measured in thirty embryos explanted at stage 4^+ to stage 5 and cultured by three different methods. Ten embryos were cultured by the New (1955) technique, ten embryos were grown on agar/albumen, epiblast side down, and ten embryos were cultured by the New (1955) technique but with the distal part of the *area opaca* excised (Fig. 1C).

The results are summarized in Figs 10–12. In embryos cultured by the New (1955) technique (Fig. 10), the *area pellucida* elongates at a mean rate of

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Fig. 7. Embryo displaying 'Pattern (a)' of abnormal segmentation. A single wide 'rod' is visible (arrows) on either side of the axis (reflected light photograph of living embryo, ×20).

Fig. 8. Embryo displaying 'Pattern (b)' of abnormal segmentation. Multiple rows of somites are present extending laterally away from the axis of the embryo in a typical 'bunches of grapes' pattern. (A, B) whole mount of embryo stained with alcoholic cochineal (A, ×15; B, ×50). (C) section through the same embryo showing the multiple somites. *epi*: epiblast; *en*: endoderm. ×45. Note the tears in the epiblast, caused by the strong attachment to the agar substrate, in this and other sections.

Fig. 9. Embryo displaying a less severe form of 'Pattern (b)' combined with wide somites ('Pattern (a)'). The two anteriormost left somites are duplicated, whilst the remaining somites on both sides are wide. (A) reflected light photograph of the living embryo. The line indicates the level through which the section in Fig. 9C was made. (×15). (B) whole mount of the same embryo after staining with light green. (C) section through the same embryo, showing the laterally duplicated somites (s). (×50). *np*: neural plate; *n*: notochord; *en*: endoderm.

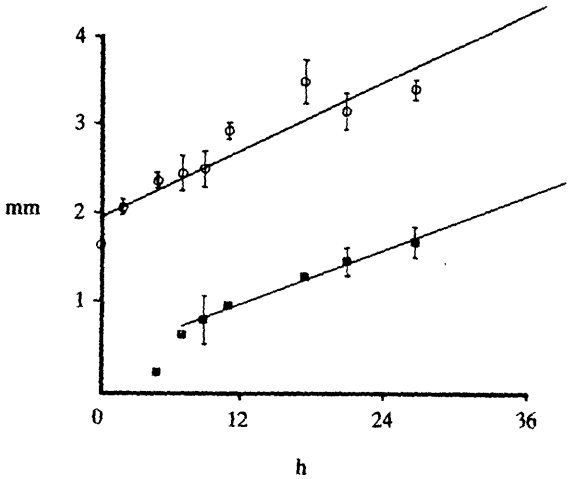

Fig. 10. Rates of elongation and regression in embryos in New (1955) culture. The abscissa represents time, the ordinate length of *area pellucida* (circles) or distance from somite 2 to Hensen's node in mm (squares). The bars represent standard error of the mean.

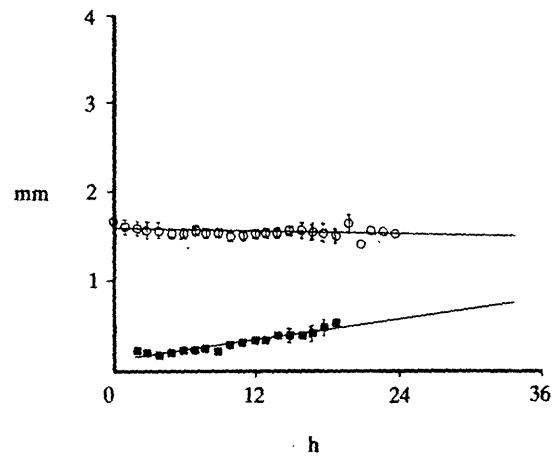

Fig. 11. Rates of elongation (circles) and regression (squares) in embryos cultured on agar/albumen. Compare rate of elongation with that in Fig. 10. Axes and symbols as for Fig. 10.

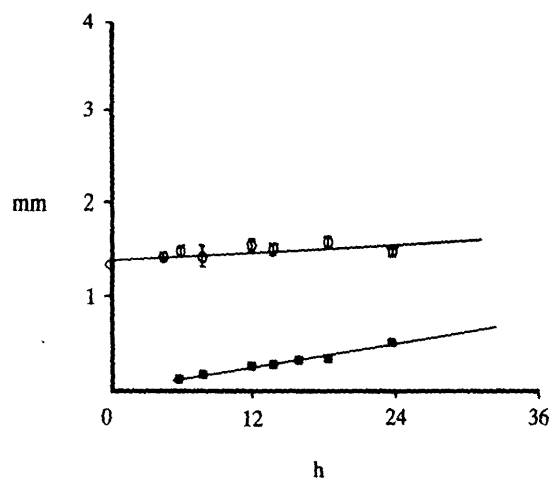

Fig. 12. Rates of elongation (circles) and regression (squares) in embryos cultured in New (1955) culture after excision of the edge of the *area opaca* as shown in Fig. 1C. Axes and symbols as in Fig. 10.

126 μm/h, but this is reduced to 17 μm/h in embryos with part of the *area opaca* removed (Fig. 12), whilst in embryos cultured on agar/albumen (Fig. 11) elongation does not take place at all.

When the rates of change in the width of the *area pellucida* were compared, no significant differences were found between rates displayed by embryos cultured by the three techniques. The average rate of decrease in width was 47 μm/hr.

The rates of regression of Hensen's node (determined from the distance between the posterior border of somite 2 and the primitive pit) averaged 102 μm/h in

New (1955) culture, 43 μm/h in embryos with part of the *area opaca* excised and 38 μm/h in agar/albumen culture.

Other observations

Some embryos have been found which spontaneously lacked a notochord (e.g. Fig. 4A, B). These embryos are characterized by having a single, medial row of somites.

DISCUSSION

Experiments of shearing through the mesoderm

Lipton & Jacobson (1974*b*) reported that if the primitive streak was sliced longitudinally (as in Fig. 1B), the somites formed 'simultaneously rather than in the normal anteroposterior progression'. They stated that their 'most convincing observations . . . come from time-lapse cinematography'. In repeating their experiments, however, we have never obtained these results but have consistently found that somite formation takes place in an orderly cephalocaudal progression. Meier & Jacobson (1982) recently carried out a similar, though not identical, experiment to that of Lipton & Jacobson, but do not appear to have obtained somites developing simultaneously; the discrepancy with the earlier report is not discussed.

We do not understand how Lipton & Jacobson (1974*b*) obtained their results since few experimental details are given in their paper. The discrepancy between their results and ours may be due to differences in operative technique or culture conditions, or to differences in the interpretation of the time-lapse films. We have sometimes observed that if there is evaporation of fluid from the dishes, the image suddenly becomes more distinct and contrasty, revealing structures which were not previously clearly distinguishable; if this occurred during Lipton & Jacobson's filming experiments, it might have led to the impression that the somites formed simultaneously.

Whatever the explanation, however, we must now conclude that *the results of our own experiments lend no support to the concept that shearing through the mesoderm releases the 'somite forming capabilities already present' in the primitive streak or that it determines the timing of somite formation.* (We will not discuss here the problem of whether cutting with a knife through all three germ layers is a true simulation of node regression). Further evidence to support our conclusions comes from two sources. First, the experiments in which a thin strip of paraxial mesoderm was removed from one side of the embryo. This experiment is also a test of whether the shearing of the presumptive somitic mesoderm from the mesoderm still in the primitive streak region controls the timing of somite formation. According to the conclusions of Lipton & Jacobson (1974*b*), we would have expected somites in the experimental side to form simultaneously. However, somite formation took place in the normal anteroposterior sequence

and at the same rate as in the control side. Second, the naturally occurring embryo (Fig. 4) in which no notochord is present, and in which therefore the mesoderm has not been sheared into right and left halves, nevertheless, has developed a row of somites. Similar embryos have been obtained experimentally after extirpation of Hensen's node by Grabowski (1956).

In criticizing the conclusions of Lipton & Jacobson (1974*b*) we do not query that the regressing node does indeed normally shear the mesoderm into right and left sides (Lipton & Jacobson, 1974*a,b*). As indicated by notochordless embryos, such a separation probably establishes the bilateral symmetry of the somites at this time (Bellairs, 1980), but it does not appear to control the timing of segmentation.

What then controls the timing of segmentation?

In the normal, unoperated embryo there is an apparent correlation between the timing of several morphogenetic events. Thus, shortly after the primitive streak has fully formed (about stage 4 of Hamburger & Hamilton, 1951) Hensen's node regresses (i.e. migrates toward the posterior end of the primitive streak), laying down a rod of notochord along its path. On either side of the notochord, the segmental plates appear. More cells are continually added to the posterior end of each segmental plate, the posterior border of each plate thereby keeping pace with the regression of the node. Meanwhile cells are removed from the anterior end of each segmental plate as somites periodically separate from it. There is evidence (Bellairs, 1980) that in the normal embryo, the future somite cells become determined soon after entering the segmental plate, but about 20 h elapse before these cells become segmented.

These considerations, together with the results of the shearing experiments reported by Lipton & Jacobson (1974*b*) led to the concept (Bellairs, 1980) that one of the roles of the regressing node was to set the 'clock' for segmentation, so that groups of cells segmented about 20 h after the node had passed them. Since we have been unable to confirm the results of Lipton & Jacobson, however, we must now modify this concept.

We suggest instead that it is not the regression of the node itself that controls the timing of the segmentation process, but certain other aspects of regression. In the normal embryo, regression not only leads to the formation of the notochord, but simultaneously to the gradual disappearance of the primitive streak as cells migrate from it. Bellairs (1984) has suggested that it is this very act of leaving the primitive streak and entering the segmental plate that starts the 'clock' for segmentation. Evidence comes from the observation (Bellairs & Veini, 1984) that isolated segmental plates continue to segment from anterior to posterior.

Regression is normally accompanied by elongation of the *area pellucida*, though as we discuss below, the two processes appear to be dissociable.

Abnormal patterns of segmentation

The results obtained with unoperated embryos cultured on agar/albumen are puzzling. No previous author has, to our knowledge, reported similar findings with their control embryos even though the culture technique was essentially the usual one in the U.S.A. throughout the 40's, 50's and 60's.

The exact mechanism by which the culture conditions provoke the abnormal somite patterns is not clear. Our results show that direct contact between the embryo and the surface of the agar/albumen is required for the abnormal patterns to be expressed. Indeed, we have found on attempting to free embryos from the surface of the substrate during fixation, that they adhere so strongly that it is often impossible to separate the two without damage. Our results also indicate that one of the processes which is inhibited in embryos cultured on agar/albumen is the elongation of the *area pellucida*, and that this is different from regression movements (see below).

Agar may chemically affect the development of embryos, as it is known (see Dixon, 1981) that agar contains mitogens and other factors affecting morphogenesis. It is also known (Dixon, 1981) that autoclaving (121 °C) can release inhibitory factors which are not produced in significant amounts by boiling at 100 °C. However, the abnormal patterns do not occur if the embryo is separated from the agar by an intervening vitelline membrane. A diffusible inhibitory factor released by agar is probably not involved because the vitelline membrane is unlikely to act as a significant permeability barrier (Bellairs, Harkness & Harkness, 1963). As direct contact between the *area pellucida* and the substrate is required, an alternative explanation might be sought at the mechanical level. Spratt & Haas (1965) reported that direct contact between the embryo and the semi-solid substratum could inhibit morphogenetic movements.

The very observation that these abnormal patterns can form in embryos contributes some interesting information about the process of normal somite formation in birds. It confirms the observation (Bellairs, 1963, 1979) that adjacent neural tube and/or notochord are not essential for somite formation, as somites can form in these embryos in regions very distant from the notochord–neural tube–primitive streak axis.

The multiple rows of somites which form in some embryos appear to arise by the fragmentation of the initial wide rods. It appears therefore that the wide somites are unstable. By contrast, the somites which form from them are within the normal size range. The histology of both the wide and the normal-sized somites is similar and corresponds with that described for normal, unoperated embryos (see Bellairs, 1979) in that it consists of a simple epithelium arranged around a small lumen. During fragmentation of the wide somites therefore, an epithelial cylinder separates into epithelial rosettes. We do not know what are the factors which bring this about. It has been suggested that local tensions within the embryo can influence the size and shape of somites (Packard, 1978; Packard &

Jacobson, 1979; Bellairs, 1979). Whatever the mechanism however, it seems likely that there is an optimum size range for the somites. This observation lends support to earlier suggestions (see Packard, 1978; Packard & Jacobson, 1979; Bellairs, 1979) that *somite-size and shape are probably controlled by local cell interactions within or near the somites*, rather than as a result of global control by the embryo.

The number of somites which form immediately alongside the axis of the embryo (i.e. the most proximal row) (see Fig. 8) is comparable both in normal embryos and in embryos with multiple rows. This finding lends support to the idea that the mesoderm is already predetermined for a certain number of somites along the segmental plate (Meier, 1979; Bellairs & Veini, 1984). In the abnormal embryos, the somites are initially wider and often turn into multiple rows so that many more somites form. Since the wide somites tend to segment into somites of normal appearance, this suggests that neither the width of each somite nor the total number of somites are inflexibly predetermined. The pre-patterning may therefore only be a 'coarse' or preliminary allocation of material, whilst the final shape of the somites and their number, may depend upon the local properties of the interactions between the cells at each somitomere. The nature of such interactions is still unknown, but there is evidence suggesting that cell–cell adhesion may be involved (Bellairs, Curtis & Sanders, 1977), perhaps via changes in the composition of surface sugar moieties (Stern, Bellairs & Durston, in preparation).

Elongation versus *regression movements*

Our measurements of the rates of elongation of the *area pellucida* and of regression of Hensen's node in New (1955) and agar/albumen cultures show that in embryos cultured by the latter method, elongation does not take place, even though regression occurs, albeit at a reduced rate. This observation indicates that *elongation and regression movements are two different events*. The measurements also indicate that *the axial arrangement of the pattern of somites may depend upon the mechanical tensions in the area pellucida*, but may not be dependent on the rate of regression.

It is of interest that somites are able to form even when the *area pellucida* does not elongate, since a comparable situation exists in amphibians; Elsdale & Davidson (1983) reported that somites could form in stunted embryos of *Rana* in which extension of the body had not occurred.

The tension exerted by the expanding edges of the blastoderm (see New, 1959; Bellairs, Bromham & Wylie, 1967; Downie, 1976) appears to be required for normal rates of elongation of the *area pellucida* and of regression, as indicated by the results of our experiments of removing half the width of the *area opaca* followed by culture on vitelline membranes.

Stability of the segmented somites

Our results, like Lipton & Jacobson's, show that stability of the somites is greatest near the notochord in agar/albumen culture. Despite the increase in

stability near the notochord, however, it is clear from the cases of spontaneous notochordless embryos (Fig. 4) that *the notochord is not essential for somites to be stable in vivo*.

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