Experimental study of the formation of the bulboventricular loop in the chick

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SUMMARY

The formation of the normal bulboventricular loop (convex to the right) and the inverted loop (convex to the left) produced by the Lepori technique in chick embryos was studied. The development of the loops was recorded by means of diagrams, photographs and microscopic time-lapse photography. Electron-microscope studies were also made. The normal loop was studied by means of labelling and removal experiments on the heart tube. The results demonstrated that the fusion of both cardiac primordia is made in stage 9—in the mid-line of the embryo and that the first asymmetry of the heart tube appears in stage 10. The truncus region developed in situ directed towards the right after the fusion of both cardiac primordia, and in this region the electron-microscope study demonstrated a gradient of caudo-cephalic differentiation. In stage 10 the left caudal groove is the prospective interventricular groove, but the right caudal groove is not the right atrioventricular groove as had been stated by others. The asymmetric incorporation of both primordia begins in stage 11—, when the curvature of the loop is already developing. In the removal experiments it was evident that the different portions of the cardiac tube in situ are orientated in space independently of the whole of the loop.

The formation of the experimentally inverted loop is a mirror-image of the normal loop and appears to be originated through mechanic traction of the cardiac tube by the left splanchopleure and not by a faster displacement of the right cardiac primordia.

INTRODUCTION

The formation of the normal and the inverted bulboventricular loop has been reported in descriptive (Patten, 1922; Davis, 1927; de la Cruz, Muñoz-Armas & Muñoz-Castellanos, 1971) and in experimental (Butler, 1952; Orts-Llorca & Ruano Gil, 1967; Sissman, 1966; Lepori, 1967; Stalsberg, 1969a, b; Stalsberg & DeHaan, 1969) studies. Most of these studies have been done by observing the structure of the loop at different moments in its formation without following its development continuously from the appearance of the heart tube until the advanced stages in its curvature. Despite numerous publi-

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cations describing the formation of the bulboventricular loop, many of the
different forms that occur during normal and abnormal development of the
loop are unknown, and many descriptions, especially those of the early stages,
are incomplete (de la Cruz, Muñoz-Armas & Muñoz-Castellanos, 1971) or
questionable (Patten, 1922; Van Mierop, 1967). Consequently, the theories that
have been put forward to explain the formation of the bulboventricular loop
have not been completely satisfactory (DeHaan, 1965, 1967; Stalsberg, 1970).

Studies on the normal and inverted bulboventricular loop are not only
important for understanding the production mechanisms of some congenital
cardiopathies (de la Cruz et al. 1967, 1971, Espino-Vela et al. 1970), but also
to interpret the electrophysiology of the embryonic heart (Patten & Kramer,
1933; Patten, 1949; Van Mierop, 1967; Lieberman, 1970).

In the present study we have used a culture technique which permits the
development of a single embryo in a time-lapse chamber, from Hamburger &
Hamilton (1951) stages 8–13.

Labelling and removal experiments of different parts of the heart tube, in
the normal and inverted loop, were made in order to investigate the importance
of each of the grooves and regions of the heart tube in the development of the
bulboventricular loop. In addition, the splanchopleure was sectioned at different
stages to study some of the factors which had been pointed out as causal in
the formation of the inverted loop.

**MATERIAL AND METHODS**

The experiments were divided into two groups: (A) study of the normal
bulboventricular loop and (B) study of the inverted loop. In both groups
fertile Rhode Island hen eggs were used, incubated at 37.5 °C, humidity 86–87 %,
until Hamburger & Hamilton stages 8, 9 and 10 were reached. The embryos
were explanted and cultivated with New's technique (1955), with the endoderm
up. After the embryos were experimented on (labelling or removal of the heart
tube), they were reincubated to stage 12 or 13 in a Hot Pack incubator at
37.5 °C, 5 % CO₂ pressure, and humidity 100 %. Other embryos were placed
in a culture chamber for microscopic time-lapse photography. These embryos
were explanted by the New technique and cultured up to stage 9 and 10. At this
time they were placed in a culture chamber consisting of a Petri dish 6 cm in
diameter in which Spratt's semisolid medium (1948) was placed up to a depth
of 0.5 cm, with a cylindrical central medium-free area, which was filled with
fluid albumen. The embryo mounted on the glass ring was placed on the albumen
so that the ring and the peripheral part of the vitelline membrane rested on
Spratt's medium. Damp cotton was placed around the ring and the culture
chamber was covered with aluminum foil with a center hole about 8 mm in
diameter for the microscopic objective (Fig. 1). The embryo was not covered
with mineral oil. Before being filmed the embryo was reincubated 0.5 h in the
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Microscope objective
Embryo
Glass ring
Damp cotton
Agar albumen

Thin albumen
Vit. membrane
Aluminum foil
Petri dish

Microscope warm stage

Fig. 1. Culture chamber for time-lapse microscopic movie camera.

Hot Pack incubator. The embryos developed normally during 48 h and in some instances reached stage 16 or 17.

Four, 8 or 16 frames per minute with ×37 enlargement were taken with a Zeiss microscopic time-lapse chamber which includes a thermostatic chamber that keeps a temperature regulated between 37.5 and 39.5 °C.

A. Study of the normal bulboventricular loop (to the right)

The formation of the normal bulboventricular loop was studied by means of labelling and removal experiments of the heart tube. Its development was recorded by diagrams, photographs and microscopic time-lapse photography. Electron-microscope studies were also made.

1. Labelling experiments. These experiments were divided into two groups:
(a) Study of the cephalic portion of the bulboventricular loop. This group comprised 25 stage-9 embryos in which the fusion site of both primordia was exposed by means of dissection of the yolk-sac membrane with fine glass needles with longitudinal cuts on both sides of the embryo joining them at the level of the subcephalic fold. Afterwards the membrane was folded towards the caudal part. Two small particles of iron oxide were taken with fine glass needles and embedded, one on the most cephalic part and the other on the most caudal part of the fusion line of the two primordia (Fig. 2A).

In 25 embryos only the vitelline membrane was dissected and a normal bulboventricular loop developed.
(b) Study of the caudal part of the bulboventricular loop. This comprised 25 stage-10 embryos with a dissection similar to the previous experiment and with an iron oxide label in the right atrio-ventricular groove and another in the prospective left interventricular groove (Fig. 3A).

2. Removal experiments. In 25 stage-10 embryos the heart tube was exposed the same way as in previous experiments. The fused part of the two primordia was then removed by a transverse cut from the right atrio-ventricular groove to the prospective left interventricular groove and by another transversal cut under the subcephalic fold, separating the portion of the heart tube included
between these two cuts by means of a fine glass needle, without damaging the underlying endoderm (Fig. 4A).

3. **Electron microscope.** The cephalic end of the heart tube in a stage-12 embryo was studied in serial sections following the longitudinal axis of the trunco-conus.

**B. Study of the inverted bulboventricular loop (to the left)**

The inverted bulboventricular loop produced by microsurgical techniques (Lepori, 1967) was studied by labelling experiments of the heart tube and bilateral cutting of the splanchopleure.

1. **Labelling experiments.** By following Lepori's technique for producing inverted loops, 25 stage-8 embryos were cut longitudinally and paramedially at the right splanchopleure from the proamnion to the level of the first somite just outside the precardiac mesoderm. The embryos were reincubated until stage 10 and an iron oxide label was placed in the right atrio-ventricular groove and another in the prospective left interventricular; the latter through a hole in the splanchopleure (Fig. 7A).

2. **Bilateral cut in the splanchopleure.** To clarify the cause of the production of inverted loops, the right splanchopleure was cut in 25 embryos using the same technique and the same stage as in previous experiment. When the embryos reached stage 10 a similar cut was made in the left splanchopleure.

**RESULTS**

**A. Study of the normal bulboventricular loop (to the right)**

1. **Labelling experiments.** (a) Study of the cephalic portion of the bulboventricular loop. The embryo was labelled at stage 9 in the site of fusion of both primordia (Fig. 2A). Upon reaching stage 12 the cephalic label was located in the right edge of the heart tube in the groove which separates the truncus from the bulbus cordis (Fig. 2C). The caudal label was also located in the right edge, but more caudal in the bulbus cordis. The results were constant and the small variations in the location of the labels was secondary to small morphological differences of the loop. In the embryos followed by microscopic time-lapse photography, it was observed that a first manifestation of bending to the right occurred in stage 10+ and 11−, revealed by a prominence of the right edge, under the caudal label in the middle and upper third of the heart tube (Fig. 2B).

(b) Study of the caudal part of the bulboventricular loop. Labels placed in stage 10 in the right atrio-ventricular grooves and in the prospective left interventricular (Fig. 3A) behaved differently in stage 12. The label placed in the prospective left interventricular groove underwent a great shift, being located in the interventricular groove or left bulboventricular loop. The label placed in the right atrio-ventricular groove shifted very little, remaining in the
Fig. 2. Location and fate of the labels placed in stage 9-. (A) Stage 9-. The labels are located in the fusion site of both primordia. (B) Stage 10+. The right cephalic edge is very prominent and the labels are already included in the cardiac tube. (C) Stage 12. Final location of the labels.
Fig. 3. Location and fate of the labels placed in stage 10 in the normal bulboventricular loop. (A) Stage 10. The labels are located in the caudal grooves. (B) Stage 11—. The shift of the labels has been symmetric, although the bending has already begun. (C) Stage 12. Final location of the labels.
Fig. 4. Removal experiment of cardiac tube in stage 10. (A) Stage 10 embryo after removing the cardiac tube. (B) Stage 11 - . The tube which has formed in the caudal region is directed toward the right. (C) Stage 12. Both the cephalic and caudal parts of the cardiac tube are directed toward the right, joined by a bridge of endocardium covered by cardiac jelly.
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Fig. 6. Higher magnification of a myocardial cell from the heart tube of stage-12 chick embryo. The large nucleus (N) shows a prominent nucleolus (n). A large, regular deposit of glycogen particles (Gl) occupies most of the cytoplasm, where a few myofibrils (Mf) begin to differentiate into a sarcomere. Specimen preparation as for Fig. 5. × 21000.

 neighbourhood of this groove, but in a dorsal position due to the development of the bulboventricular loop (Fig. 3C).

In the two embryos in which the shift of the labels was followed by microscopic time-lapse photography, the final results were identical, but it could be observed that the shift of both labels up to stage 11 – was symmetrical (Fig. 3B). From this time on the label placed on the left side moved more rapidly.

Fig. 5. Electron micrographs of a myocardial cell from the cephalic region of the heart tube of a stage-12 chick embryo. The cytoplasm is occupied by irregular deposits of glycogen particles (Gl), rough endoplasmic reticulum cisternae and irregular aggregates of contractile myofibrils (Mf), which are mostly cut in transverse or tangential views. At the luminal region of the cell, adjacent plasma membranes differentiate to form specialized intercellular junctions such as intermediate junctions (IJ) or desmosomes (D). N = nucleus. This cell exemplifies an intermediate stage of differentiation in the cephalic region of the cardiac tube at stage 12. × 5400. Specimen fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, embedded in Epon. Stained with lead citrate and uranyl acetate.
Fig. 7. Location and fate of the labels placed in inverted loop in stage 10. (A) Stage 10. The labels are located in the caudal grooves. Note that the anterior portal gut and the cardiac tube are displaced toward the left. (B) Stage 11. The labels have shifted symmetrically. (C) Stage 12. Final location of the labels. Note the elongation of the loop.
2. **Removal experiments.** The caudal part (primitive ventricle) and the cephalic end (truncus region) of the loop developed in all the embryos, both directed to the right and joined by a bridge of endocardium (Fig. 4 A–C). This endocardium was covered by cardiac jelly which was demonstrated histologically.

3. **Electron microscopy.** Examination of the cephalic end of the heart tube under the electron microscope gave the following results. A progressive differentiation of myocardial cells in the caudal direction was observed. This was reflected in the progressive appearance of several cytoplasmic constituents. One of the most prominent was the presence of differentiated myofibrils forming in mature sarcomeres, which were found only in the most caudal regions of the heart tube. The presence of well-defined sarcomeres was in general accompanied by the existence of intercellular junctions at the luminal end of adjacent plasma membranes, in the form of desmosomes and intermediate junctions (Fig. 5). In general it was observed that the amount of glycogen deposits in myocardial cells was inversely related to the presence of sarcomeres and intercellular junctions, i.e. those cells which were least differentiated, located near the cephalic end, were those that had more prominent deposits of glycogen granules (Fig. 6).

B. **Study of the inverted bulboventricular loop (to the left)**

1. **Labelling experiments.** In stage 12 all of the loops were convex to the left and concave to the right (inverted loops), but most of them were rather elongated in comparison to the normal loop. The labels shifted in an approximately mirror-image compared to the labels placed in the normal loop. The label placed in the left side always ended up near the left atrio-ventricular groove, but underwent a slight cephalic shift greater than that of the label placed in the right side of the normal bulboventricular loop (Fig. 7C). In the embryos followed by microscopic time-lapse photography the same facts were observed as in the right loop, but in a mirror-image (Fig. 7B).

2. **Bilateral cut of the splanchopleure.** Twenty-two embryos developed a normal loop (to the right) and 3 developed an inverted loop (to the left).

**DISCUSSION**

It is generally considered that the fusion of the cardiac primordia occurs in a cephalo-caudal direction, beginning in the truncus and ending in the primitive atria. Our experiments demonstrate, however, that the fusion of the two cardiac primordia in stage 9 — occurs at the level of the conus and that the truncus develops *in situ* posteriorly. Labels placed in stage 9 — at the cephalic end of the region where the cardiac primordia had already fused (Fig. 2A) were subsequently found to lie caudal to that end of the heart tube corresponding to the truncus (Fig. 2C). These same results are evident by observing marks d and b in Stalsberg & DeHaan’s (1969) figures 11 and 12, although the authors
did not comment about them. In the experiment in which the heart tube was removed in stage 10 (Fig. 4A) the truncus always developed in the cephalic part directed caudally and to the right (Fig. 4B, C). The electron-microscope study showed the existence of a caudo-cephalic differentiation gradient; the most differentiated parts are those nearest the conus (Fig. 5). Labelling experiments demonstrate that the truncus develops from the most cephalic part of the cardiogenic areas.

The labels placed in the right atrio-ventricular groove and in the prospective left interventricular groove show that the atrio-ventricular is not the definitive-groove. This confirms the findings of Stalsberg & DeHaan (1969) and indicates that the final groove develops posteriorly (stage 11—).

The removal experiments make clear that in the formation of the bulbo-ventricular curve the whole heart tube is not necessary but that each of the parts can form the corresponding portion of the loop and that it is normally orientated. It has also been shown that the cephalic and caudal ends of the loop intervene independently in its formation.

The labels placed in the caudal grooves in stage 10 in embryos operated on for inverted loop (Fig. 7) shifted approximately in a mirror-image to the labels located in the same regions in the normal loop (Fig. 7B, C). The slight differences in the shifting of the labels are explainable by the greater elongation of the loops. Just as in the normal loop, the asymmetric movement of both labels began in stage 11—, when the formation of the bulboventricular curve had already begun. This indicates that the beginning of the bending is independent of the asymmetric incorporation of both primordia, but the faster incorporation of the right primordium is the cause of the accentuation of the inverted loop and the mirror-image behaviour of both primordia, especially in the caudal part.

Lepori (1967) pointed out that the cause of the bending of the bulboventricular loop can be interpreted thus: 'Under normal conditions, the latero-medial movement of the left splachnopleure is faster than that of the right one and the meeting of both cardiac primordia takes place as a result, in a right medial plane instead of in the mid-sagittal plane. The C bending movement of the left cardiac primordium also precedes that of the right, so that, at the moment of the fusion, the rear extremity of the left cardiac C inserts itself into the concave part of the right cardiac C, producing the typical right bending of the cardiac tube.' The same author suggests that the inverted loop is due to a delay of the movements of the left splachnopleure or to an acceleration of the movements of the right one, which leads to a relatively faster movement of the right heart primordium, and thus fusion occurs in the left paramedial plane and the posterior part of the right C inserts itself into the concavity of the left C. Nevertheless, we have proved by means of histological sections and by observation of fresh embryos that in stage 9— and 10 the fusion of both primordia occurs in the mid-line.
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When the right splanchopleure in stage 8 was cut, an inverted loop developed in practically all of the embryos, according to Lepori (1967), due to the fact that the right primordium reaches the mid-line first. However, when the right splanchopleure was cut in stage 8 and the left in stage 10 in the same embryo, in 88% of the cases a normal loop developed. This fact cannot be explained according to Lepori’s hypothesis, since in stage 10 both primordia are fused to a great extent and the formation of the inverted loop has begun, which should continue its development as such despite cutting the left splanchopleure.

As soon as one of the splanchopleures is cut, the anterior intestinal portal shifts to the opposite side of the cut, and the same thing happens later with the embryonic axis. This indicates that when the right splanchopleure is cut, the traction exercised by the left splanchopleure on the forming heart tube facilitates the greater incorporation of the right primordium in the caudal region, from stage 11—, a phenomenon opposite that which occurs in the normal loop. When this traction is stopped in stage 10, by cutting the left splanchopleure, the loop becomes normal. This experiment demonstrates that formation of the normal or inverted loop is not due to an asymmetric fusion of both primordia, nor to the different C curvature of each of them, since although they are fused and supposedly curved, the type of loop is still not determined because the direction of the loop can be changed experimentally.

CONCLUSIONS

The results obtained in the experiments reported in this paper lead to the following conclusions:

1. The fusion of both heart primordia is made in stage 9— in the mid-line of the embryo and not in the right paramedial as had been indicated.
2. The first appearance of asymmetry of the heart tube is at stage 10, when the cephalic portion of the right edge becomes more convex.
3. In stage 10 the left caudal groove is actually the prospective interventricular groove as Stalsberg & DeHaan (1969) have already indicated, while the right caudal groove is not the right atrio-ventricular groove as has been stated, but rather this appears in stage 11—.
4. The labels placed in the caudal grooves in stage 10 shifted symmetrically until stage 11—. From that time on the shift of the label of the left caudal groove is faster. This fact coincides with accentuation of the bulboventricular curve because of the faster incorporation of the left primordium in the caudal part.
5. In the removal experiment the truncus region developed in situ directed toward the right. The same thing happened when labels were placed in the cephalic end of the heart tube in stage 9—. In the caudal part the primitive ventricle region always developed toward the right, and joined the cephalic portion through a bridge of endocardium. This demonstrates that the different
parts of the heart tube in situ are orientated in the space independently of the whole of the loop.

6. As soon as one of the splachnopleures is cut, the anterior intestinal portal shifts toward the opposite side of the cut, and the same thing happens later with the embryonic axis.

7. The labels placed in a stage 10 in the caudal grooves of the inverted loop shifted in a mirror-image to the normal loop. This indicates that when the right splachnopleure is cut the traction exercised by the left splachnopleure on the forming heart tube increases the speed of incorporation of the right primordium in the caudal region from stage 11—.

8. When the right splachnopleure in the same embryos was cut in stage 8 and the left one in a similar extension in stage 10, in 88% of the cases a loop was formed on the right. This fact cannot be explained by Lepori's hypothesis since in stage 10 formation of the inverted loop has begun and should continue developing as such despite cutting the left splachnopleure.

9. It can be concluded from these experiments that in the normal loop there are two differential growth processes which succeed one another in space and time, one on the right side of the cephalic portion in stage 10 and the other on the left side of the caudal portion from stage 11— on, although the innermost causes of their origin are unknown. In the inverted loop produced experimentally, there is a differential growth process in the right caudal region from stage 11— on.

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REFERENCES


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