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Commentary

Commentary on "The precardiac areas and formation of the tubular heart in the chick embryo" by Stalsberg and DeHaan, 1969

Developmental biologists, like fans of superhero legends, love a good origin story. It's captivating to follow our favorite cells as they arise from their humble beginnings to attain their fantastic destinies, and it's intriguing to consider how their paths were shaped by the environments encountered en route. Fate mapping approaches, in which cells are marked in the early embryo and then later detected once they reside in a particular tissue, reveal the beginning and end of a developmental story and allow us to formulate hypotheses regarding the trajectories followed in between. Moreover, fate map data can inspire models for the regulatory mechanisms that direct uncommitted cells toward their ultimate destinations.

Fifty years ago, many developmental biologists were eagerly applying classical fate mapping techniques in an effort to understand the cellular underpinnings of pattern formation within a variety of embryonic organs. This approach, and its value, is admirably exemplified by the work of Stalsberg and DeHaan (1969), who focused on the fate map of the precardiac mesoderm in the chick embryo. Prior studies had identified a pair of bilateral "heart-forming areas" in the early chick embryo, and Stalsberg and DeHaan aimed to acquire a refined understanding of their dimensions and organization. Importantly, they wanted to determine where the precursors of different sections of the heart, such as the conus, ventricle, and atrium, resided within these bilateral areas. Were distinct subsets of precursors strictly organized or frequently intermingled? Detection of a pattern within the precardiac mesoderm would have interesting implications for the morphogenetic manner through which the bilateral fields of precursors become an organized organ at the embryonic midline.

To construct their fate map, Stalsberg and DeHaan chose to transplant small squares of tissue from various locations in radiolabeled donor embryos into the equivalent positions of unlabeled host embryos at stage 5. The next day, when the embryos had reached stage 12, they evaluated the positions of the transplanted cells, relative to a carefully defined atlas of 24 subregions within the heart tube. By comparing the initial and final sites of each transplant, they concluded that the precursors of distinct regions of the heart are relatively organized within the precardiac mesoderm, with a general correspondence between the rostrocaudal axis of the heart tube and the positions of the precursors. This organization of the cardiac fate map suggested that cardiac morphogenesis might proceed in a highly coherent fashion, without much intermixing of the myocardial precursors. Indeed, additional work performed by Stalsberg and DeHaan – including systematic microdissection and sectioning of the mesoderm, as well as tracking embedded iron oxide particles over time – suggested that the precardiac mesoderm behaves as a cohesive sheet of tissue throughout the process of heart tube formation.

Collectively, the work of Stalsberg and DeHaan, together with other contemporaneous fate mapping efforts, provided a fundamental framework for envisioning cardiac morphogenesis as an orderly process in which an organized sheet of precursors "condenses, stretches, folds, and deforms" to give rise to a patterned heart tube. Over the following decades, as techniques for fate mapping and lineage tracing have become increasingly sophisticated, the field has continued to build on this foundation, both by reinforcing the primary message regarding early patterning of the precardiac mesoderm and by enhancing our understanding of the diversity of cardiac lineages and the complexity of their organization (Evans et al., 2010; Meilhac and Buckingham, 2018). Additionally, the advent of a variety of strategies for high-resolution imaging have provided new opportunities to track cardiac cell behavior and to evaluate its regulation by intrinsic properties of the mesoderm as well as external influences of neighboring tissues (Cortes et al., 2018; Varner and Taber, 2012). Even so, many mysteries remain regarding the molecular mechanisms that direct cardiac cell fate and control the coherence of cardiac morphogenesis. Thus, the classic experiments of Stalsberg and DeHaan still resonate today, as we continue to seek the regulatory principles that underlie their "unified concept of heart formation and tubulation".

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The Precardiac Areas and Formation of the Tubular Heart in the Chick Embryo[1]

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INTRODUCTION

Numerous previous studies have been concerned with the location of heart-forming material in the presomite chick embryo. This literature has recently been reviewed (DeHaan, 1965, 1969). The best-known map of the boundaries of the precardiac mesoderm in the chick is probably that of Rawles (1943), who determined the developmental capacities of fragments, isolated from the head-process stage embryo, which were grown as chorioallantoic grafts. She found tissue with heart-forming potency in two broad oval areas, one on each side of the primitive node, separated by a median noncardiac zone about 0.4 mm wide. More recent studies, both biochemical (Ebert, 1953, 1955; Duffey and Ebert, 1957; Chiquoine, 1957) and morphological (DeHaan, 1963a, b; N. LeDouarin, 1964), have lent support to this concept of two separate heart-forming areas at the head-process stage.

The method which is potentially most accurate for mapping the prospective embryonic fate of tissues is that of transplanting radioactively labeled tissue fragments from a donor embryo to the homologous site in an unlabeled recipient embryo. The subsequent fate of the implant can then be determined by autoradiographic analysis of serial sections of the embryo at a later stage of development. This technique was applied to the presomite chick embryo by Rosenquist (1966), and by Orts-Llorca and Collado (1968). From the embryos in which labeled cells were found in the tubular heart, Rosenquist and DeHaan (1966) were able to determine where the prospective heart mesoderm had been

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in the head-process embryo. As in the previous mapping studies, they found bilateral precardiac areas. However, they also indicated that these areas were bridged across the midline, rostral to the prechordal plate, by a narrow zone of prospective endocardial and conal myocardial cells. They concluded that even in the head-process embryo, the precardiac mesoderm was organized not as separate, paired, heart-forming regions, but as a cardiogenic crescent (Mollier, 1906).

The accuracy of the study of Rosenquist and DeHaan was limited by the size and position of their implants. The implanted fragments were 0.3–0.4 mm wide, the most medial of which were positioned to abut on the midline, but not to extend across it. Thus these fragments were too large to permit recognition of a median cardiac-free zone smaller than about 0.6–0.8 mm wide. It was therefore desirable to extend this study specifically to test the existence of a median bridge of heart-forming material. This was the first aim of the present work.

In an earlier study DeHaan (1963a,b) employed time-lapse cinematography to study the behavior of the precardiac mesoderm. He concluded from the appearance of cell groups on the films that the heart-forming material moved as independently migrating clusters, within the splanchnic mesoderm. These movements were reported as random between stages 5 and 6, becoming oriented and coordinated from stage 6+ until the clusters were lost in the opacity of the forming heart tube at stage 9–10. However, Rosenquist and DeHaan (1966) observed that fragments implanted at stage 5 retained their integrity as coherent masses of tissue throughout further development of the heart. The implanted fragments became folded, stretched and distorted in shape as they contributed to the epimyocardial tube. But tritium-labeled epimyocardial cells did not intermingle with those of the surrounding heart wall, as would have been predicted from the independent migration of small cell clusters. They concluded that the precardiac splanchnic mesoderm moves and folds as a cohesive sheet in the formation of the epimyocardium.

If this conclusion is accurate, then sharp boundaries must exist in the precardiac mesoderm at the head-process stage between preconal, preventricular, and preatrial tissues. Indeed, any defined region of the later heart tube must have its sharply outlined counterpart in the precardiac areas. Therefore, the second goal of the present work was to map the regional subdivisions of the precardiac mesoderm in the head-process stage embryo. The method employed for this was similar to that of Rosenquist and DeHaan (1966). However, the size of the implants

was reduced, the pattern of implantation was concentrated in and near the presumed cardiogenic crescent, and both sides of the embryo were utilized for implants.

Finally, the nature of the mesoderm as a coherent sheet, and the changes of form that this sheet undergoes during heart formation were confirmed by microdissections of whole cultured embryos at closely spaced stages of development, by graphic reconstructions of serially sectioned embryos, and by a series of experiments in which the forming epimyocardial walls were marked with particles of iron oxide. From these studies a unified concept of heart formation and tubulation has been developed.

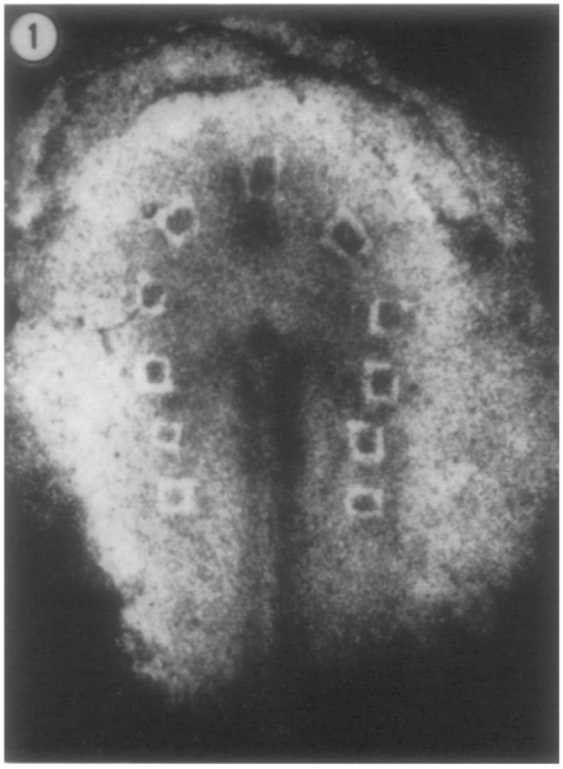

FIG. 1. Stage 5 donor embryo. Eleven fragments of endoderm-mesoderm have been cut and are ready to be explanted. Ten additional fragments were later dissected from the spaces remaining, forming a continuous crescentic row of 21 explants, transferred in symmetrical pairs to 11 hosts (one receiving only the rostral midline implant). × 30.

METHODS

Transplantation Experiments. Fertile DeKalb eggs were incubated at 37.5°C for about 20 hours and then explanted to watch glass cultures, endoderm side up, by the technique of New (1955). Developmental stages were determined according to Hamburger and Hamilton (1951). All transplants were performed between embryos at stage 5 (5^- to 5^+).

Tritiated thymidine (0.83 mC/ml; specific activity 15.9 Ci/mmole) dissolved in 70% ethanol was diluted 1:100 in Howard-Ringer saline (Howard, 1953). A 10 μl drop of this solution containing 0.08 μC of tritiated thymidine, was applied to the endodermal surface of early stage 5 embryos selected as donors. Fourteen control embryos were similarly treated at stage 4–6 with 0.08 or 0.83 μC of tritiated thymidine and incubated to stage 11–13 without further treatment.

At the end of a 2-hour labeling period (at 37.5°C) the endodermal surface of the donors was washed several times with Howard-Ringer saline. Hooked glass needles were used to cut several square fragments from each embryo according to one of several preconceived patterns

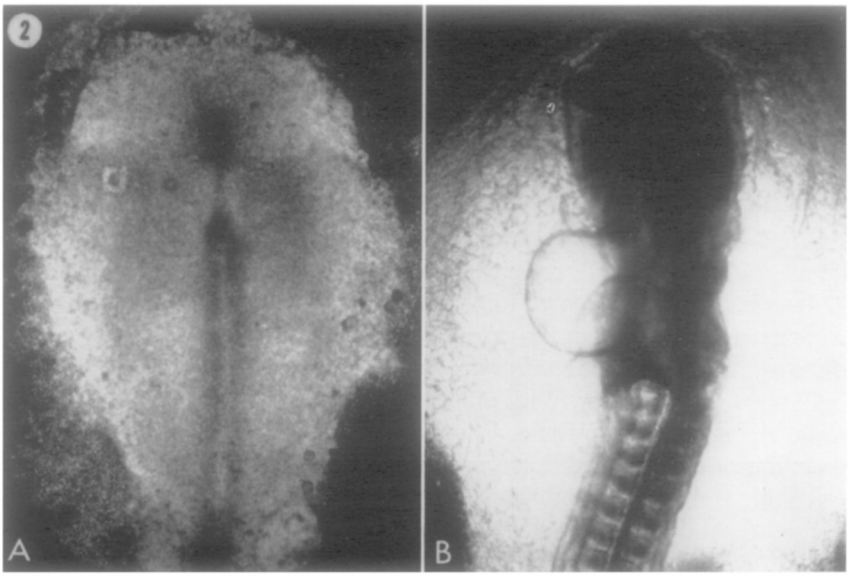

FIG. 2. Host embryo after implantation of a single small labeled fragment. (A) Stage 5. Immediately after operation. The fragment has adhered, but has not yet begun to heal in. (B) Stage 12. Twenty-four hours after implantation, photographed just prior to fixation to show normal heart development. × 22.5.

(Fig. 1). In the rostral areas, where no mesoderm was present at this stage, the fragments consisted of endoderm only. Otherwise, each fragment included endoderm and mesoderm. The dimensions of these squares varied from 0.1 to about 0.2 mm. A small cut was made near one corner of each square to maintain orientation.

Each donor embryo was photographed on Polaroid film, and the photograph was used as a guide for positioning the implant in the recipient. In unlabeled recipient embryos one or two fragments corresponding in size, position, and composition to the donor fragments were cut out and discarded. Each donor fragment was rinsed three times in each of two dishes of sterile saline, and transferred to the recipient. It was fitted into the prepared bed in the host and lightly pressed down for a few seconds (Fig. 2A). In the experiments in which each host embryo carried two labeled implants, they were positioned one on each side of the embryo in such a way that the two implants were not expected to contribute to neighboring areas of the heart. The position of each implant always corresponded to the site of origin of the fragment in the donor, as precisely as this could be achieved by visual comparison.

After transplantation, recipient embryos were photographed on Polaroid film and reincubated at 37.5°C for about 24 hours, to stage 11–12 (Fig. 2B). The embryos were fixed in formalin, embedded, and sectioned at 7 μ. The sections were mounted on slides and deparaffinized. They were then coated with Kodak NTB2 liquid emulsion according to the methods of Prescott (1964). The preparations were exposed at room temperature in light-tight boxes for 12–14 days, developed, and stained with Ehrlich's hematoxylin and eosin. For this transplantation study 15 donors and 77 recipient embryos were prepared and studied.

Graphic Reconstructions. From the serial sections a ventral view graphic reconstruction of each experimental recipient embryo was made, as follows. The notochord axis was drawn as a straight vertical line on millimeter graph paper. With an ocular micrometer, the lateral distances from the notochord to the contours of the heart and foregut, and to the boundaries of labeled cell areas, were measured (usually on every fifth section), and this measurement was transferred to the graph paper. The longitudinal dimension was determined by the distance between sections and converted to the same scale as the lateral dimension. Finally, corresponding points from sequential sections were connected with smooth lines to delineate the outlines of the heart and foregut and the position of labeled cells.

Graphic reconstructions of sectioned untreated embryos, fixed at

intervals between stage 5 and stage 12 were also made, by a slightly different method (Greenfield, 1968), to examine the normal configuration of the mesoderm layer during this period.

Dissections. Morphologically normal embryos cultured by the New technique (1955) were selected at closely spaced intervals between stage 5 and stage 12. To the endodermal surface of an embryo was added 1–3 drops of 1% trypsin (dissolved in calcium- and magnesium-free saline). After 1–3 minutes, when the endoderm could be separated from mesoderm, one drop of embalming fluid[3] was added to stop the tryptic action and lightly fix the tissues. With two fine tungsten needles, both the yolk-sac endoderm and foregut were removed from the area under study. In older stages the anterior part of the neural tube was cut away to facilitate visualization of the mesoderm. The preparations were photographed at several stages during the dissection procedure, and detailed drawings were made.

Marking with Iron Oxide Particles. Normal embryos, cultured by the New technique, were selected at stage 9–10, and transferred, on the vitelline membrane, to the surface of a semisolid agar-albumin medium[4] (DeHaan, 1963a). Each embryo was flooded with Ringer's solution. With fine tungsten needles, under the layer of saline, small holes were torn through the yolk-sac and ventral body wall, exposing the forming heart tube and portions of the epimyocardial troughs. Particles of insoluble ferric oxide were inserted through the holes and pressed on or into the epimyocardial wall, positioning them usually at the anterior and posterior ends of the formed conoventricular tube, and at two or three points in the undifferentiated cardiogenic material. Excess saline was carefullly sucked off and the preparation was reincubated for an hour to allow the particles to adhere firmly and the torn tissues to begin healing. The embryo was then washed to remove nonadhering particles and flooded with nontoxic mineral oil[5] to prevent evaporation and maintain optical clarity. Embryos maintained at 37.5°C on a microscope warm-stage incubator were photographed at hourly intervals with a 10-second pulse of motion-picture film (24 frames/second), or every 4–6 hours with high-speed Polaroid film. In the latter case, still-pictures of the beating heart were taken in mid-diastole.

[3] Embalming fluid (per liter) 95% ethanol, 100 ml; glycerol, 70 ml; phenol, 50 ml; formaldehyde (40%), 50 ml; Howard-Ringer saline, 730 ml.

[4] Agar-albumin medium. 1 gm Noble agar, 1 gm dextrose, 25 ml Howard-Ringer saline, 75 ml fresh egg albumin.

[5] "Klearol," Sonneborn Division, Witco Chemical Co., New York.

At the end of the culture period the embryos were fixed in 4% buffered formol, stained with alcoholic cochineal, cleared, and prepared as whole mounts for examination at higher magnifications.

OBSERVATIONS

Tritium-Labeled Implants

Of the 14 control embryos, 12 (86%) developed completely normally. Two exhibited dextrocardia. These results are not different from those obtained with untreated cultured embryos, and are interpreted as indicating that neither the thymidine-^{3}H nor the ethanol in which it was dissolved had deleterious effects on embryos at the dilutions at which these agents were applied.

In experimental embryos observed 2–4 hours after operation, implant tissue could usually no longer be distinguished from the surrounding host tissues. Among the 77 operated recipients, the heart and foregut were determined by inspection to have developed normally in 67. These embryos carried a total of 103 labeled implants. In three of the recipient embryos, no labeled cells could be found in the autoradiographed serial sections. The distribution of labeled cells in the host tissues from the remaining 100 implants is summarized in Table 1.

On the autoradiographed serial sections, labeled cells were found either in both endoderm and mesoderm, or in endoderm only. With only a few exceptions the grafted tissue was fully integrated into host structures, and was microscopically indistinguishable from surrounding host tissue except for its radioactivity. In embryos with labeled mesoderm, graft

TABLE 1

DISTRIBUTION OF LABELED FRAGMENTS IN HOST TISSUES AT STAGE 12

Position of labeled region	Number		
Epimyocardium alone	3		
Endocardium alone	7		
Epimyocardium and endocardium	45		
Total in heart		55	
Noncardiac mesoderm alone		28	
Mesoderm plus endoderm			83
Mesoderm alone			0
Endoderm alone			17
Total implants			100

cells were usually found in both somatic and splanchnic layers. When label was present in mesoderm and endoderm, there was a characteristic rostrocaudal sequence: labeled cells in the somatic mesoderm were more rostral than those in the splanchnic layer, while the labeled region of endoderm, whether in the yolk sac or foregut, was most caudal.

Epimyocardium. Forty-eight implants contributed labeled cells to the stage 12 epimyocardium. To test the existence of a rostromedian bridge of premyocardial cells, 11 implants were made directly in the midline, rostral to the notochord. Another 15 were placed in paramedian positions extending up to 0.4 mm lateral to the midline. None of these 26 fragments contributed labeled cells to the epimyocardium. Thus, the preepimyocardial mesoderm is clearly organized at stage 5 in two separate regions, with a gap of about 0.8 mm between them.

In confirmation of the previous findings (Rosenquist and DeHaan,

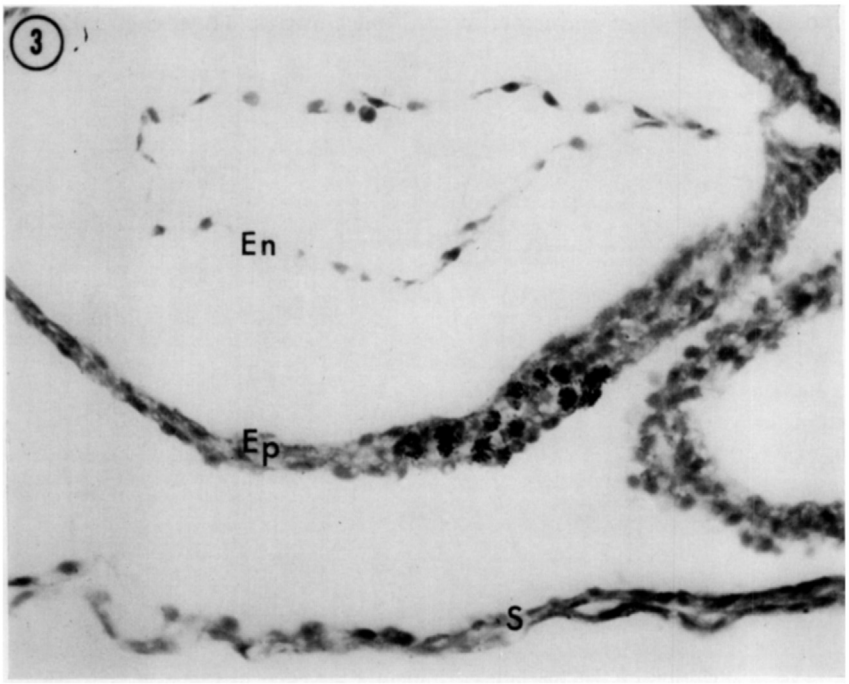

FIG. 3. Autoradiographed section through the ventricle of a stage 12 host embryo, illustrating the labeled implant in the epimyocardium with typical well-defined boundaries. *En*, endocardium; *Ep*, epimyocardium; *S*, splanchnopleure. × 410.

1966), labeled cells in the formed epimyocardium were always found in well defined areas (Fig. 3), as elongate bands running obliquely to the axis of the heart. Within a graft region, 25–50 % of the cells were usually labeled in the section. Labeled and unlabeled nuclei were uniformly distributed throughout the labeled band, with no indication of fewer labeled cells near the edges of the labeled area. The findings thus confirm that in the epimyocardium there is no significant intermingling between the cells of the labeled implant and those of the surrounding host tissue.

Further evidence in support of this contention is derived from experiments such as that illustrated by the donor embryo in Fig. 1, in which a series of implants were made, each contiguous with the last (although, of course, each in a different host embryo). When the resulting labeled areas of epimyocardium in the stage 12 heart tube were plotted together on a single drawing, the fit between adjacent areas was remarkably good, despite some gaps and overlap that could reasonably be ascribed to individual variation and experimental inaccuracies. Thus, each implant

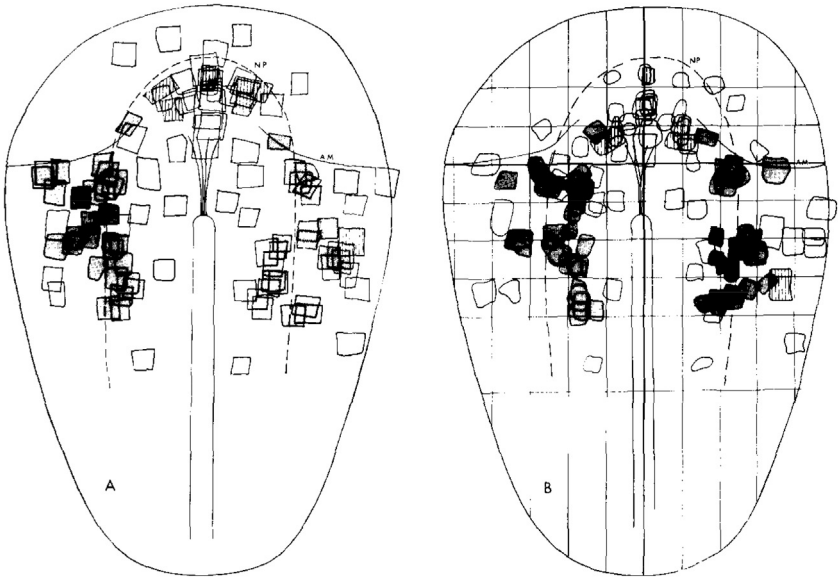

FIG. 4. Position of the 103 labeled implants included in this study. (A) Map made by visual comparison of the postoperative photograph (as Fig. 2A) with the standardized map embryo. (B) Map made by using the superimposed grid lines for points of reference. Implants which contributed cells to both epimyocardium and endocardium are indicated with heavy shading; to endocardium only, with vertical hatched shading. *NP*, neural plate; *AM*, anterior edge of mesoderm.

appears to fulfill the developmental task of the excised piece it replaces. Any defined region within the epimyocardium at stage 12 can be traced back to an equally well-defined region in the stage 5 mesoderm, from which apparently, all of its progenitor cells arise.

To determine the boundaries of the subdivisions of the precardiac mesoderm it was necessary to synthesize observations made on different embryos which exhibited substantial variation in size and shape. For this

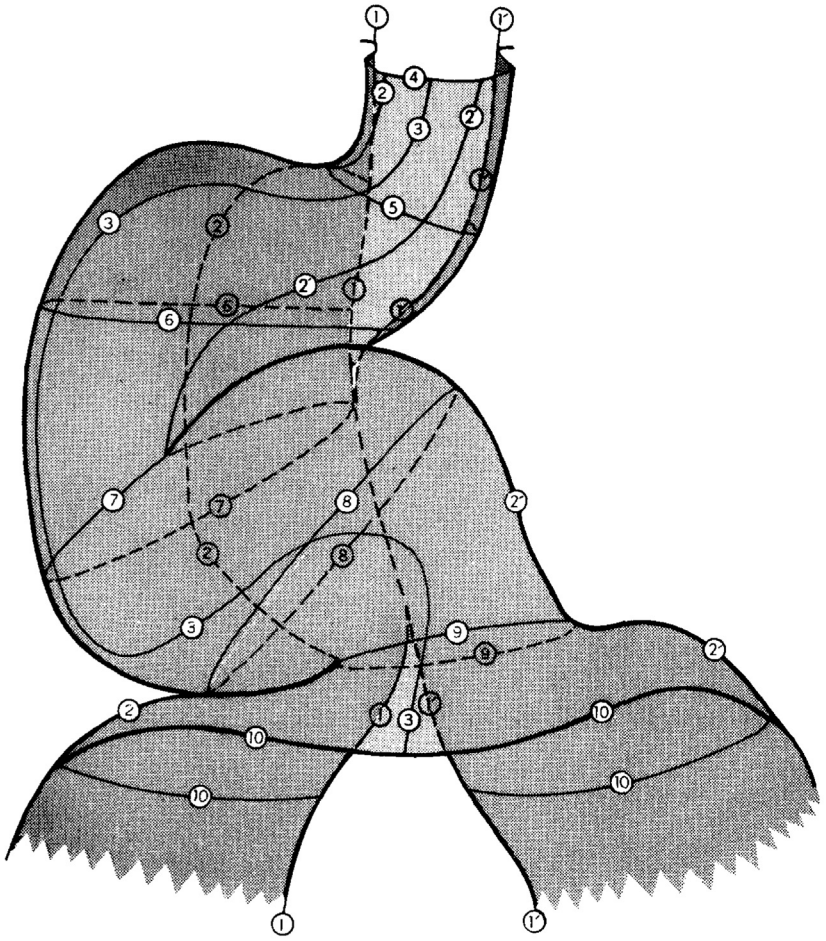

FIG. 5. The heart-tube (epimyocardium only) subdivided into 24 defined regions by transverse and longitudinal lines. The intersections between these lines were taken as reference points.

purpose, a semischematic standardized average stage 5 embryo was drawn. The position of each of the 103 implants was transferred from the individual postoperative photographs of the recipient embryos (Fig. 2A) to the drawing of this average embryo. Two such map-plots were made, employing different methods for estimating the position of each fragment. In one map (Fig. 4A) the position of each fragment was plotted simply by visual comparison between the standardized map embryo and the photograph of each recipient embryo. In the second map (Fig. 4B) a grid pattern was drawn on the standardized map embryo. A corresponding grid was drawn on a glass plate, of a size suitable to fit an average recipient embryo as photographed. This glass plate was placed over each recipient photograph, using the midline of the embryo and the anterior edge of the lateral plate mesoderm as reference structures to position the grid. The implant was then plotted on the map according to its relation to the grid.

Each mapping method had certain disadvantages. The first was more susceptible to errors of subjective measurement. The second tended to disregard individual variations in size and shape of the embryos. Therefore, mapping analysis was carried through with both maps, and the results were averaged.

For mapping purposes a standardized reference stage 12 heart was also drawn (Fig. 5). The heart tube was divided into 24 subdivisions, defined by a set of five longitudinal lines (numbered *1*, *1′*, *2*, *2′*, and *3*) and seven transverse lines (numbered *4* to *10*). Lines *1* and *1′* define the attachment of the heart to the dorsal mesocardium on each side. Line *3* represents the ventral line of fusion between the left and right heart-forming regions (Stalsberg, 1968). Lines *2* and *2′* run midway between these dorsal and ventral boundaries. The intersections between these longitudinal and transverse lines were the main points of reference for mapping.

To illustrate the mapping procedure the determination of the position of the intersection between lines *2′* and *6* is shown, slightly schematized, in Fig. 6A, B. From the graphic reconstructions of the sectioned and autoradiographed recipient embryos, four embryos were found to carry labeled areas in the epimyocardium that included the reference point (*2′*, *6*). The positions of the four labeled areas are shown by the contour lines *a*, *b*, *c*, and *d* in figure 6A. On the implant map (Fig. 4A) the corresponding implants were found to have been positioned as shown in Fig. 6B. From the location of the reference point (*2′*, *6*) within the labeled area *a* (Fig. 6A), it appeared that the cells which gave rise to that intersectional point had been near the center of the implant *a* (Fig. 6B).

Thus the point of presumed origin of these cells is indicated by the star in the center of implant *a* (Fig. 6B). In the labeled area *b*, the reference point is caudal and ventral to the center of the labeled area. From preliminary plots, the general representation of dorsoventral and rostrocaudal directions in the stage 5 embryo was known. On this basis, the second estimate of the position of point (*2′*, *6*) on the implant map was as shown by the star within implant *b*. Similarly, the third and fourth estimates of the reference point were derived from implants *c* and *d*. For the average embryo, the best estimate of the origin of the cells at point (*2′*, *6*) was taken as the geometrical midpoint between the four individual estimates, shown by the circle in Fig. 6B. In statistical terms, the lines connecting the midpoint to each of the individual estimates show the scatter of the individual observations around their mean.

This process was repeated for each of the 21 intersections on each side,

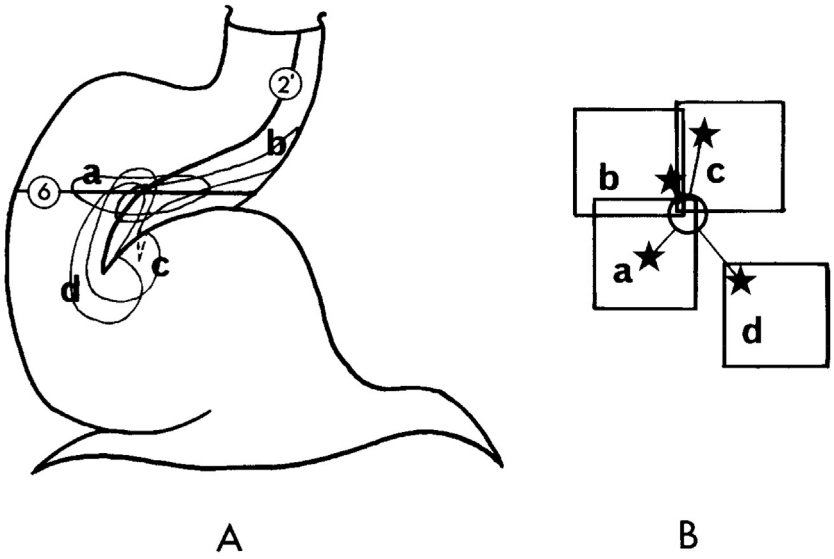

FIG. 6. The method of mapping a reference point from the epimyocardium at stage 12 on the precardiac area at stage 5. (A) Semischematic drawing of a stage 12 heart, including reference lines *2′* and *6* of Fig. 5. Areas (*a*, *b*, *c*, *d*) of labeled epimyocardium have been transferred from graphic reconstructions of four different embryos to this drawing. (B) The relative location on the implant map (Fig. 4A) of the four implants from which the labeled areas in Fig. 6A were derived. The stars show the four individual estimates of the point of origin of the cells that end up at the intersection (*2′*, *6*) at stage 12. The circle marks the geometric midpoint of the four individual estimates.

resulting in the "star-map" shown in Fig. 7. On this same map are also plotted the positions of implants which lay just outside or inside the outer boundaries of the heart-forming regions, or which included part of those boundaries without contributing cells to an intersection. These were used as secondary aids in positioning those boundaries. A second star-map (not shown) was then produced by repeating this process for the implants, as positioned in Fig. 4B, and these two star-maps were averaged. By connecting the averaged intersectional points with smooth lines, the reference lines of Fig. 5 were reconstructed—i.e., projected—on the stage 5 reference embryo (Fig. 8).

The two heart-forming areas and their regional subdivisions thus determined (Fig. 8) are similar in general size, shape, and position. The transverse segments of the heart tube are represented by curved bands of precardiac tissue on both sides. There is some asymmetry apparent in the size of the corresponding regions on the two sides. The rostral three regions are larger on the embryo's right side, and the sum of the caudal three regions is larger on the embryo's left side. However, considering the scatter of the individual estimates of each reference point, one cannot exclude the possibility that these differences arose from errors of the method.

Endocardium. Of the 48 preepimyocardial implants, all but three also contributed labeled cells to the endocardium (Table 1). In addition, seven gave rise only to endocardial cells. The latter were mainly located in the rostromedial region. One of the 11 rostral midline grafts contributed two endocardial cells to the tubular heart. Among the remaining median and paramedian implants, the most medial ones that contributed cells to the endocardium extended about 250 μ from the midline on each side. Thus, except for the two midline cells the preendocardial areas were ordinarily separated by a gap of about 0.5 mm. The endocardial contribution from the one midline fragment may represent an occasional prechordal plate contribution to the endocardium in normal development, or it may be an abnormal finding caused by experimental manipulation of the embryo.

The position of the labeled cells in the endocardium corresponded roughly to the position of labeled cells in the epimyocardium. However, the structure of the endocardium made it difficult to judge whether cells derived from an implant had remained together as a group, or to what extent they may have intermingled with host-derived endocardial cells. In the epimyocardium, where the cell nuclei were closely packed, the boundary of each graft area could be readily identified even though

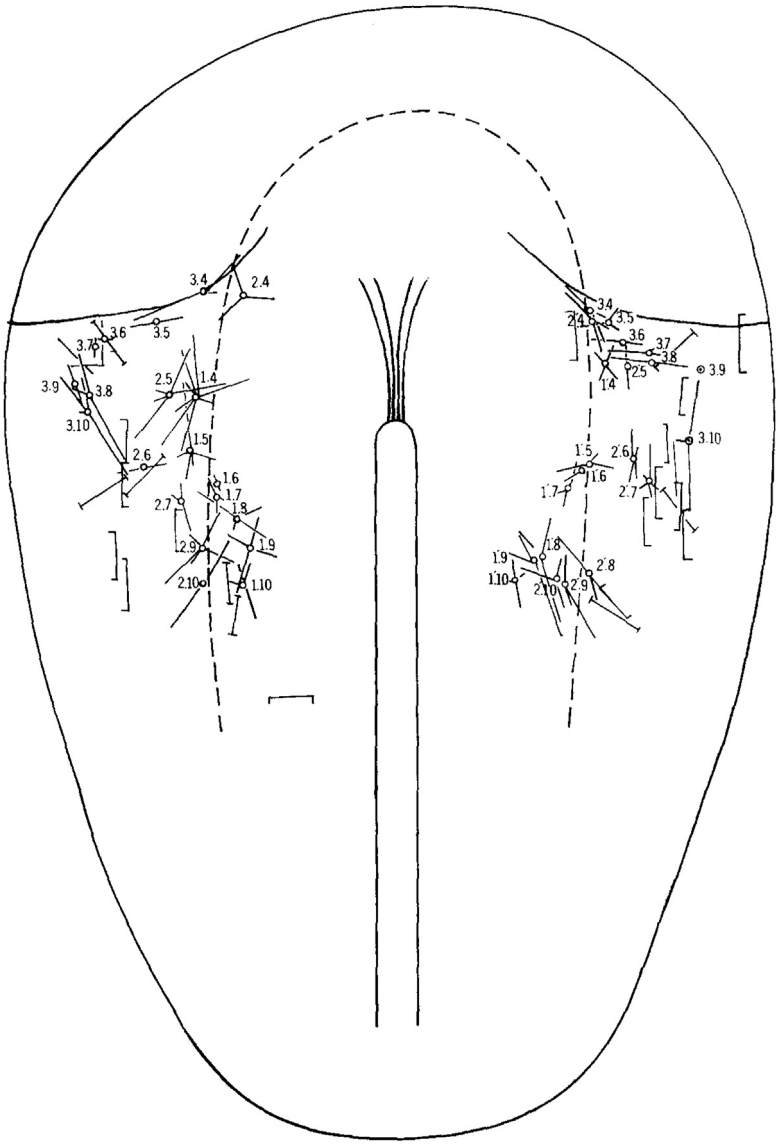

FIG. 7. Estimates of the position in the stage 5 mesoderm of the cells which form the intersectional reference points shown in Fig. 5 determined as illustrated in Fig. 6.

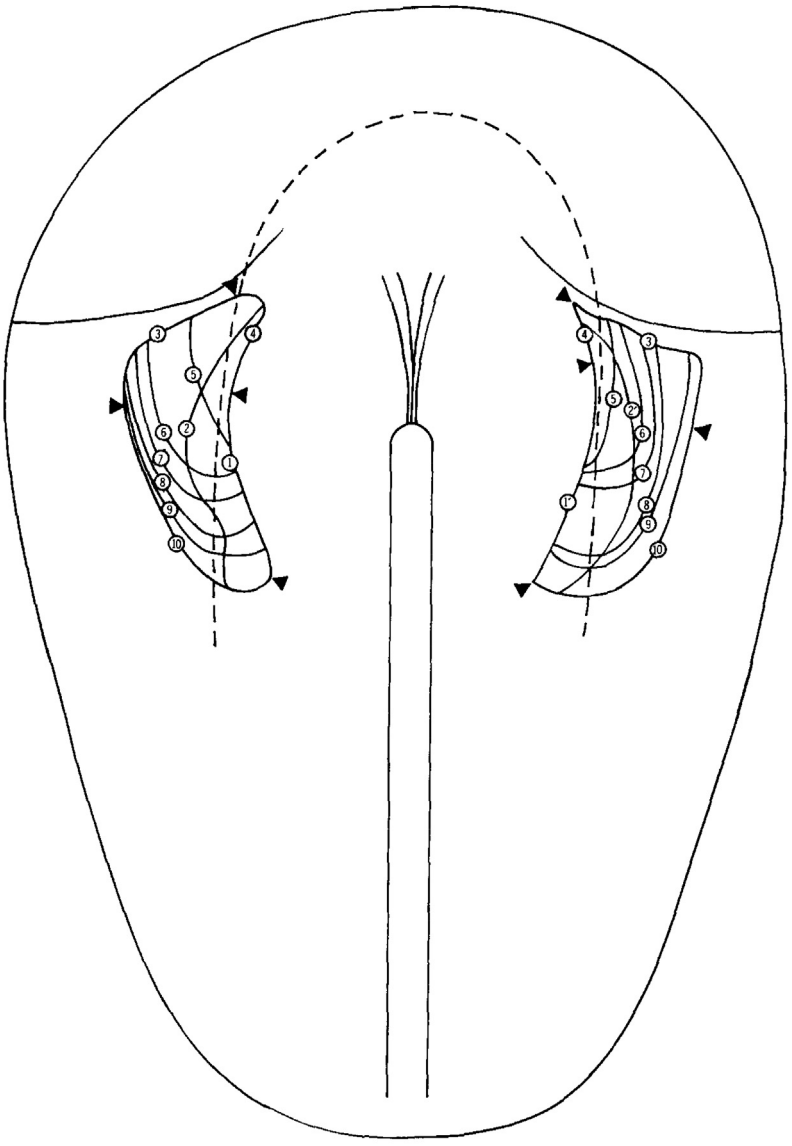

FIG. 8. The boundaries and subdivisions of the preepimyocardial areas in the stage 5 embryo. This map is constructed from the averaged data of two star maps: the one shown in Fig. 7, based upon the implants as positioned in Fig. 4A; and a similar one based upon the implants as positioned in Fig. 4B. The arrowheads on the outer boundary of each precardiac area designate the ends of the boundary lines (lines *1* (*1'*), *4*, *3*, and *10*).

50–75 % of the cells within the area were unlabeled. In the endocardium, however, the distance between neighboring nuclei was large. The presence of a high proportion of unlabeled graft cells thus made it impossible to outline accurately the graft-derived area as had been done for preepimyocardial cells.

For this reason, a map of preendocardial regions was constructed by a less rigorous method than that described above. As a reference, the stage 12 heart was subdivided by transverse lines into only six regions (Fig. 9A; regions *a–f*). Each fragment of the implant-map (Fig. 4A) was then evaluated as having contributed endocardial cells to one or more of the regions. A smooth line was drawn through the center of all the implants which had contributed to region *a*, all those which contributed to region *b*, and so on, yielding a regional map of preendocardial fields (Fig. 9C). A similar map was made for preepimyocardial fields by the same method (Fig. 9B).

The subdivisions of the precardiac areas, mapped in this way, show extensive overlap. Nonetheless, there is still a distinct rostrocaudal sequence of regions in both epimyocardium and endocardium. We have

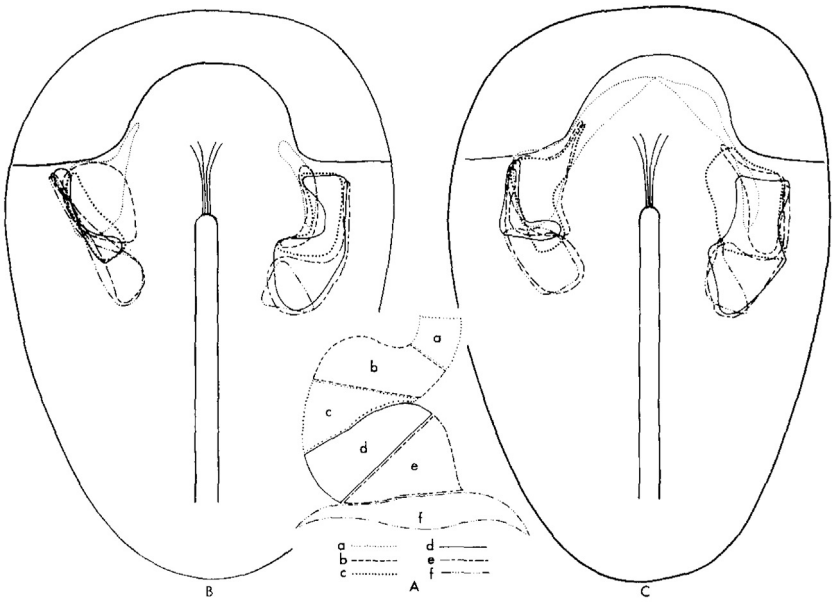

FIG. 9. Regional subdivisions of the precardiac mesoderm at stage 5. (A, Inset) The heart tube at stage 12, with 6 rostrocaudal regions indicated by letters *a–f*. (B) Preepimyocardial subdivisions. (C) Preendocardial subdivisions.

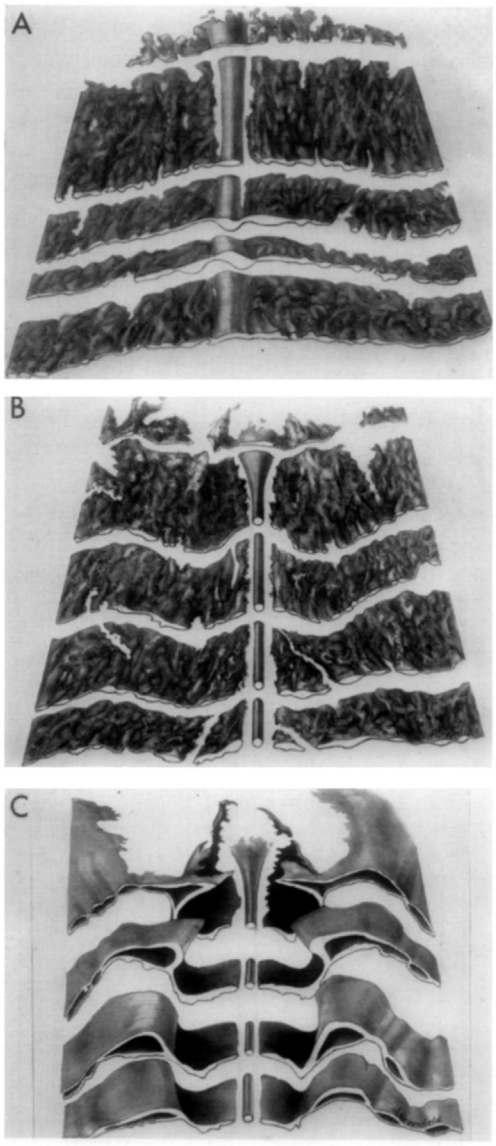

FIG. 10. Drawings of the mesoderm layer during heart formation, made from graphic reconstructions and microdissections of embryos at stages: 6 (A), 7 (B), 8 (C), 8^+ (D), 9 (E), and 10^+ (F). Endoderm and ectoderm are not shown. Forming endocardium is illustrated on embryos left side only.

assumed that preepimyocardial areas are sharply bounded, with no actual overlap in the individual embryo as depicted in Fig. 8. Thus, all the overlap appearing in Fig. 9B must be ascribed to variations among the individual embryos and inaccuracies inherent in the mapping technique. Similar factors must be responsible for at least part of the overlap of preendocardial areas (Fig. 9C). However, the preendocardial regions are even larger, and the overlap correspondingly more pro-

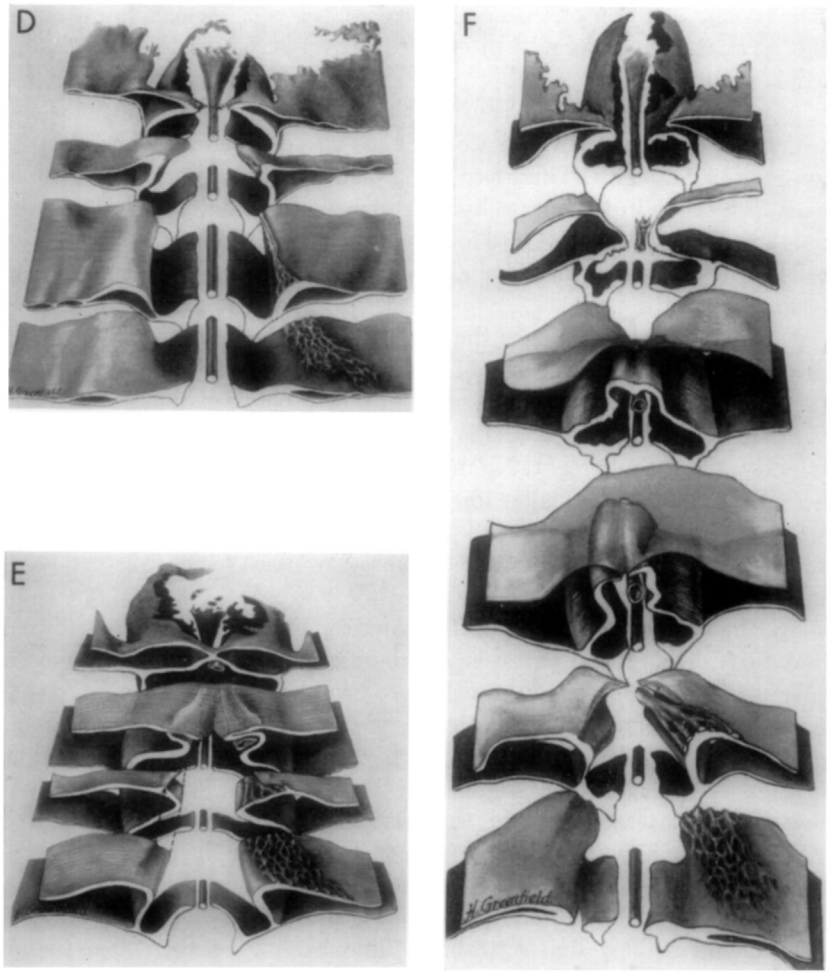

FIG. 10 D–F

nounced than in preepimyocardial regions, suggesting that in the individual embryo, preendocardial regions are less sharply bounded than preepimyocardial regions, and that intermingling of preendocardial cells from adjacent fields may take place.

Configuration of the Mesoderm Layer

The mesoderm layer of embryos at stages 6, 7, 8, 8^+, 9, and 10^+ is shown in Fig. 10A-F. Each drawing was made from a graphic reconstruction of an embryo representative of that stage. The configuration of the layer was corroborated in each case by microdissections of similar whole embryos.

At stage 6 (Fig. 10A), the lateral mesoderm forms a thin flat sheet which can be lifted with a dissecting needle as a coherent layer from the underlying ectoderm. The layer has a distinct rostral border which can usually be seen in the intact living embryo. Immediately lateral to the notochord, mesoderm cells are few and scattered. In the lateral plate, the cells form a spongy, mesothelial layer, with no pronounced regions of local condensation.

During subsequent stages the ectoderm and endoderm fold to form, respectively, the early head fold and shallow foregut. The anteromesial portion of the mesoderm folds concomitantly between these two layers (Fig. 10B) and by stage 8 (Fig. 10C) is found enclosing the roof and lateral walls of the foregut. No mesoderm (with the possible exception of occasional scattered cells) is found ventral to the foregut, since at this stage the gut floor is composed of endoderm which was initially rostral to the prechordal plate in an area (the proamnion) which was devoid of mesoderm.

By the time the embryo has 4–5 somites (Fig. 10C, D) the head fold and anterior intestinal portal have progressed caudally, and pronounced spaces, the amniocardiac vesicles, have been left between the separated layers of splanchnic and somatic mesoderm. Laterally, the rostral edge of the mesoderm has swung forward to form a broad arc in front of the head fold. Opposite the caudal end of the ectodermal head fold, where it becomes the lateral body fold, the mesoderm is thrown into a sharp fold which pushes in medioventrally from each side, over the anterior intestinal portal. This fold is usually most advanced on the right side.

At stage 9^- (Fig. 10D,E), the amniocardiac vesicles from the two sides have extended almost to the midline, leaving between them only a narrow band of mesodermal cells forming the ventral mesocardium. In the dorsal layer of the ventromedial mesodermal folds, which invest the

ventral foregut, a zone of thickened mesoderm is seen on each side of the midline, and each has formed a concavity between itself and the endoderm. These represent the first portion—the rostral ends—of the epimyocardial troughs. At their rostral ends, the concavities of the paired epimyocardial troughs are directed dorsomedially, open toward the ventral surface of the foregut floor. More caudally it is seen that each concavity has gradually rotated so that it is open medially over the anterior intestinal portal. Still farther back, the troughs complete a 180-degree torsion, their shallow concavities exposed on the ventral surface of the mesoderm caudal to the anterior intestinal portal. Within these concavities clusters and bands of hemangioblasts have appeared. These begin to hollow out to produce the primitive tubular endocardial plexus.

With further development (Fig. 10E) the amniocardiac vesicles from each side extend to the midline, break through the ventral mesocardium, and separate the ventral pericardial wall from the heart. Soon, the two epimyocardial troughs fuse to form a single, median trough which is the early conoventricular part of the heart (Fig. 10F).

At no time does the forming heart "tube" resemble a straight, closed cylinder. At stage 9 (Fig. 10E) the fused epimyocardial troughs give the heart the shape of a broad funnel open dorsally. At the lateral edges of the troughs, the epimyocardium is continuous with the mesoderm investing the floor of the foregut. By stage 10 (Fig. 10F), the reflected layers on each side are still wide apart over most of the length of the heart. As late as stage 12, the heart has a closed dorsal roof only in the midventricular region (Fig. 5), where the two reflected layers are brought into direct contact to form a dorsal mesocardium. At its rostral and caudal ends the heart "tube" is still a broad trough open dorsally and caudally against the endoderm of the foregut and AIP.

The differentiated heart elongates by continued fusion of progressively more posterior segments of the epimyocardial troughs which feed in over the ever-receding AIP. Between stages 9 and 10, constrictions or sulci become distinguishable, dividing the heart into rostral and caudal segments. By stage 10, these sulci are usually obvious, and the heart bulges distinctly to the embryo's right (Fig. 10F), presaging the later d-looping of the ventricular region.

Iron Oxide Marking Experiments

An embryo marked with iron oxide particles at stage 9⁻ is shown at four sequential stages in Fig. 11A–D. The particles designated by letters (*d*, *e*, *f*, *g*, *m*) are embedded in or adhering to the epimyocardium. Un-

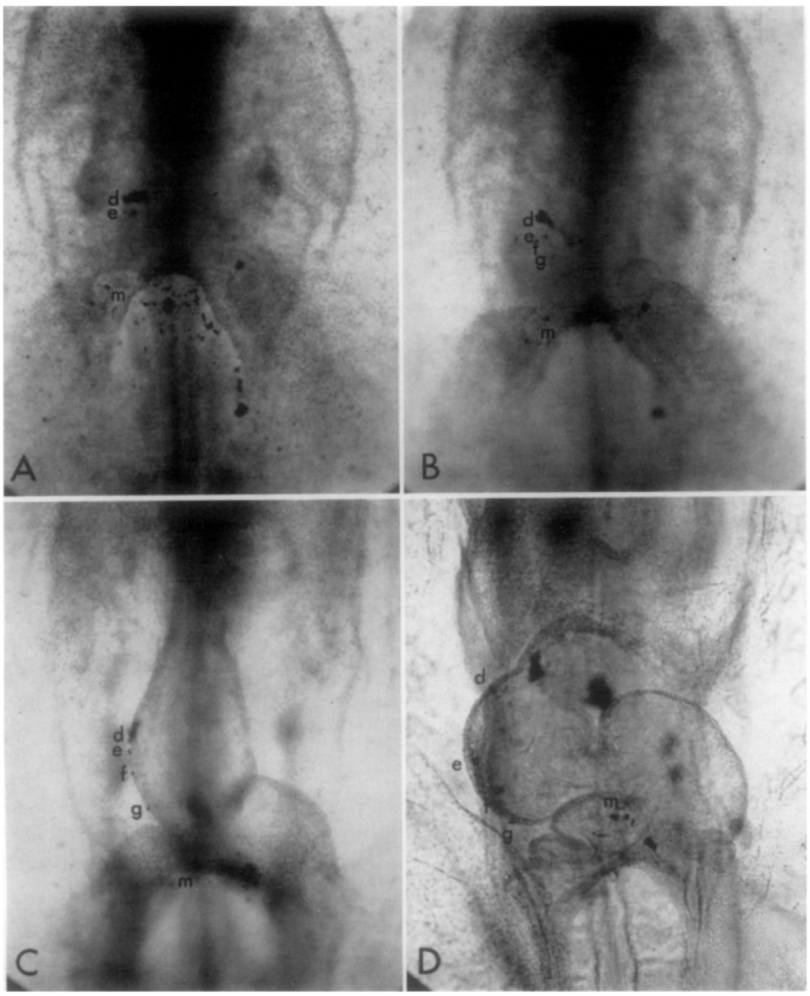

FIG. 11. Embryo marked with particles of iron oxide in epimyocardium. (A) Stage 9⁻, immediately after marking. Mark *d* is obscured by a large clump of powder lying below the heart. Marks *f* and *g* are out of the plane of focus. (B) Stage 9 (C) Stage 10 (D) Stage 12. × 66.

lettered particles are lodged in the head mesenchyme, or adhering to the overlying endoderm. The distinction between particles in the heart or elsewhere is readily made in the living embryo as soon as the heart begins to beat. Marks in or on the epimyocardium beat with it. The embryo shown is one of the series in which Polaroid photographs were taken every few hours.

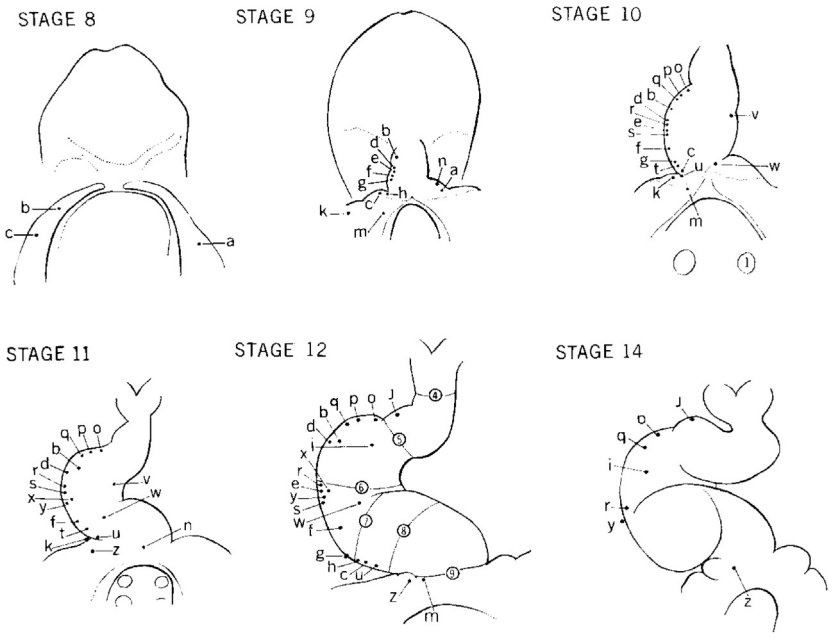

FIG. 12. Elongation and curvature of the heart tube. Each lettered point represents the position of a particle of iron oxide, photographed at three or more consecutive stages during development.

The results of the entire series of particle-marking experiments are summarized in Fig. 12. The shape of the heart is shown semidiagrammatically at intervals between stage 8 and stage 14. The position of particles attached to the epimyocardium is indicated by lettered dots. The majority of these particles were placed on the right ventral margin of the forming heart tube. During stages 10–12, as the tube undergoes torsion to the right, these particles rotate from ventral to dorsal. No indication of this rotation is given in Fig. 12. It should also be emphasized that this figure is a composite of 25 particles carried on 14 different embryos, marked or photographed at 15 stages between stages 8 and 15 No single embryo had more than a few of the particles shown on each of the stages drawn.

At stage 10 (Fig. 12), two sulci may be seen at the right lateral margin of the heart (left side on the drawing). The rostral right sulcus is frequently indistinct at stage 10 but becomes marked from stage 11. The position of this sulcus is stable relative to the marking particles (*o*, *p*, *j*) from stage 10–11. It represents the right conoventricular sulcus.

The caudal right sulcus has a different relation to the marking parti-

cles, which are seen to travel across the sulcus from the posterior epimyocardial trough material into the caudal end of the formed heart (*c*, *h*, *k*, *u*). This sulcus, therefore, marks the transition between fused and nonfused parts of the epimyocardial trough, and moves caudally as the posterior part of the trough is gradually fused with its mate from the left side, confirming that the early heart tube continues to elongate by progressive addition of material from behind.

At the left lateral margin, one deep sulcus is seen at stage 10, at the marking particle *w*. The relation between the sulcus and the particle remained constant during stages 10–12. Thus, this sulcus seems to be stable and represents the prospective interventricular sulcus. More caudally on the left side, a less distinct sulcus is present at stage 10, becoming more marked at stage 11. This apparently is also a transitional sulcus, corresponding to the one on the caudal right side. At stage 12 these two caudal sulci mark the position of the atrioventricular canal.

In addition to elongation by addition of newly differentiated tissue from behind, a second mechanism of cardiac elongation is also demonstrated by these marking experiments. Between stages 9 and 14 it is clear that particles along the right lateral margin of the heart tube gradually move apart (Fig. 12, *bc*, *defg*, *opqrstu*, *xy*). This indicates that some of the

TABLE 2

DIFFERENTIAL ELONGATION OF THE HEART, MEASURED ON PARTICLE-MARKED EMBRYOS (IN ARBITRARY UNITS)

Contour Measured	Segment	Stage			St 12/ St 9	St 12/ St 10
		9	10	12		
Axial straight line	Rostral end to AIP	14.9 ± 2.7 (8)[a]	—	44.7 ± 7.2 (9)	3.0	—
Mid-heart curvature	Rostral end to AIP	15.1 ± 3.5 (8)	—	69.4 ± 14.2 (9)	4.6	—
Right lateral contour	Rostral end to point g [b]	8.3 ± 3.0 (8)	—	52.8 ± 14.0 (9)	6.4	—
Left lateral contour	Rostral end to IV sulcus	7.6 ± 2.5 (8)	—	22.8 ± 7.2 (9)	3.0	—
Right lateral contour	Point g to right caudal sulcus	—	3.8 ± 2.2 (11)	11.1 ± 4.4 (9)	—	2.9
Left lateral contour	IV sulcus to left caudal sulcus	—	7.6 ± 4.9 (11)	38.6 ± 7.2 (9)	—	5.1

[a] Figures given are mean and standard deviation; the number in parentheses is N = number of embryos on which the measurement indicated was made.

[b] Point g is the position along the right lateral contour of the heart tube opposite the prospective IV sulcus, equivalent to particle *g* (Fig. 12).

elongation must result from expansion of tissue already differentiated as myocardium, during that period.

To quantify the observed elongation of the heart, a series of measurements was made on the photographs of the marked embryos, of the heart contours at stage 9, 10, and 12, as follows: (a) from the rostral end of the tube to the AIP in a straight axial line, (b) from the rostral end of the tube to the AIP along a line drawn down the middle of the heart, following its curvature, (c) from the rostral end of the heart tube to a position along the right margin opposite the prospective IV sulcus (equivalent to marking particle *g*, Fig. 12) termed point *g*, (d) from the rostral end of the heart along the left margin to the prospective interventricular sulcus, (e) from point *g* to the caudal right sulcus along the right lateral margin of the tube, and (f) from the prospective interventricular sulcus to the caudal left sulcus, along the left lateral margin. These measurements are summarized in Table 2. The calculated ratios indicate the relative change in length of the rostrocaudal space within which the heart forms, the length of the heart tube, and the lateral contours of the heart tube rostral and caudal to subdivision line 7.

DISCUSSION

In the stage 5 embryo the mesoderm which will form the epimyocardial layer of the tubular heart is organized into two separate regions, each with its medial edge no less than about 0.4 mm on either side of the embryonic axis. These preepimyocardial areas are substantially narrower than the fields of heart-forming potency described by Rawles (1943), as was in fact predicted by her, and demonstrated by Rosenquist and DeHaan (1966).

Both the labeled implants and the dissection and reconstruction data given here confirm that the preepimyocardial mesoderm behaves as a coherent sheet, in the formation of the tubular heart (Rosenquist and DeHaan, 1966). It condenses, stretches, folds, and deforms, but it does not lose its integrity as a sheet, nor its continuity with the rest of the layer of splanchnic mesoderm. It does not break up into cells or cell clusters which migrate independently. These findings are consistent with the electron microscopic evidence that the splanchnic mesoderm represents a mesothelial layer rather than a mesenchymatous one (Manasek, 1968). It follows, therefore, that sharply bounded subdivisions of the epimyocardium in the tubular heart should derive from equally well-defined regions of precardiac mesoderm. These regions have been mapped with no overlap on the assumption that variations in size, shape,

and position of the precardiac subdivisions and boundaries, from one embryo to another, can be represented as deviations around an average configuration (Figs. 7 and 8).

Previous studies, both morphological (DeHaan, 1963a,b; Rosenquist and DeHaan, 1966) and functional (DeHaan, 1963c; G. LeDouarin *et al.*, 1966), have indicated that the precardiac mesoderm can be subdivided along a generally anteroposterior axis. The most rostromedial portions form the conus and conoventricular regions. The caudal mesoderm produces the atria and sinus venosus of the tubular heart. The subdivisional map depicted here (Fig. 8) largely confirms this pattern of organization. However, at the lateral edges of the precardiac mesoderm, because of the curved shape of each segment, the subdivisions exhibit a mediolateral organization, preatrial tissue reaching almost as far rostrally as preconal tissue. Moreover, the subdivisional boundaries shown in Fig. 8 represent only those preepimyocardial regions which will have differentiated by stage 12. At that stage the atria are just forming; the sinus venosus has not yet differentiated. We assume, therefore, that some preatrial tissue, and all the prospective sinus venosus, is located at stage 5 lateral and caudal to line 10 in Fig. 8.

The subdivisions of the stage 12 myocardium, and the projection of those subdivisions onto the heart-forming regions of the stage 5 embryo by means of the labeled implants, has provided maps of the beginning and end of a selected segment of the cardiogenic process. It is possible to produce a continuous picture of the intervening stages in this process by sequentially distorting the regional map of the stage 5 precardiac mesoderm so that it becomes superimposable on the forming epimyocardial troughs and heart tube as seen by microdissection or graphic reconstruction of sectioned embryos (Fig. 10). We know that the tubular heart forms in an orderly rostrocaudal sequence by progressive addition of precardiac material at its posterior end (Patten, 1922; DeHaan, 1963b; Rosenquist and DeHaan, 1966). From the particle-marking experiments described above (Fig. 12) we can estimate which portions of the precardiac mesoderm enter the heart tube at each stage. We can also approximate how much of the elongation of the organ is due to expansion of already differentiated myocardium, and how much to new material feeding in from behind. With this information and the previous maps of Rosenquist and DeHaan (1966) as guides, the subdivision lines in Fig. 13 have been drawn at each stage to give a smooth and simple transition from the beginning to the end of the sequence.

From this series it becomes clear that the rostrolateral boundary of

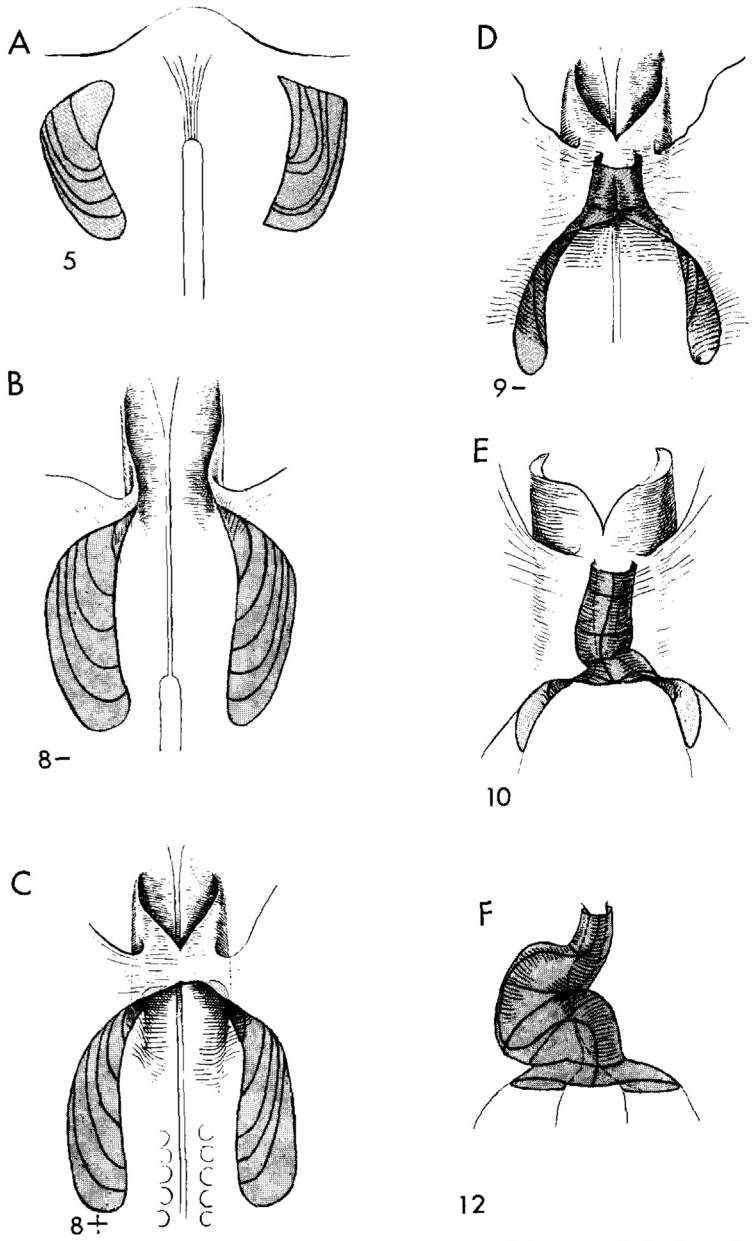

FIG. 13. Heart formation from stage 5 to stage 12. The map of the boundaries and subdivisions of the precardiac mesoderm has been appropriately distorted to be superimposed upon the forming epimyocardial troughs and epimyocardial tube, to produce a smooth transition of shapes through the intervening stages.

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each of the heart-forming regions (Fig. 13A; line *3* of Fig. 8) forms the crest of the fold of splanchnic mesoderm, which becomes the ventral edge of each epimyocardial trough. As these edges swing together and fuse, they establish the ventral midline of the heart tube and carry the subdivision lines, one after the other, into a horizontal position (Fig. 13C–F; lines *4–10* of Figs. 5 and 8).

According to measurements made of the heart contours on the photographs of the embryos marked with iron oxide particles, the curving heart tube increases in length by a factor of 4.6 between stages 9 and 12. During the same period, the straight-line rostrocaudal distance from the rostral end of the conotruncus to the anterior intestinal portal increases by about threefold (Table 2). The difference between the length of the heart and the space in which it is restricted has been considered to be the prime cause of the folding or d-looping of the heart tube (Patten, 1922). The results presented above, however, suggest that instead of mere linear elongation, the tubular heart undergoes expansion in different regions, at different rates on its left and right sides. Between stages 9 and 12 the linear distance from the rostral end of the heart to point *g* (approximately equivalent to the distance between lines 4 and 7, Fig. 5) along the right margin of the tube increases by more than sixfold. Along the left side this increase is only about threefold. On the other hand, between stages 10 and 12, there is substantial elongation of the posterior end of the heart tube (between point *g* and the caudal sulci). Along the right side this increase is about threefold; on the left side the corresponding contour increases approximately fivefold.

It should be emphasized that these measurements were made on photographs—i.e., two-dimensional projections—of the hearts. Such observations ignore completely any changes in the dorsoventral dimensions of the heart tube. They also fail to distinguish between changes in the lateral contours of the tube brought about by rotation or torsion and those caused by linear expansion. They do, however, indicate clearly that during the time the heart loops and bends to the right, it also undergoes striking differential elongations on the two lateral contours. Thus these measurements are consistent with the idea of differential expansion of the two sides, as suggested by earlier workers (Davis, 1927; Bacon, 1945; Butler, 1952; Orts-Llorca and Gil, 1967).

In a recent study of the origin of cardiac asymmetry (Stalsberg, 1968), it was found that more epimyocardial cells in the rostral portions of the heart tube are derived from the right precardiac area than from the left. In the caudal part of the heart, on the other hand, most cells are

derived from the left precardiac mesoderm. In the map of the preepimyocardial regions shown in Fig. 8, a corresponding asymmetry is seen. The total size of the regions rostral to subdivision line 7 is substantially larger on the left than on the right, while the opposite is true posterior to line 7, where the sum of the regions is greater on the right. This difference could be related to the asymmetry in cell number in the tubular heart, suggesting differential rates of incorporation of precardiac material into the heart from the two sides.

Whereas the epimyocardium derives from a cohesive mesothelial layer (Manasek, 1968), the endocardium is formed from small groups of cells which begin to emigrate from the thickened epimyocardial mesoderm into the endomesodermal space at stage 8^+ (Sabin, 1920). The mapping studies suggested that a degree of intermingling of these preendocardial cells from adjacent areas may occur. That is, preendocardial cells seem freer to move relative to one another than are premyocardial cells. It may well have been the movements of these cells that were recorded on cinema film (DeHaan, 1963b), from which the anteroposterior organization of the cardiogenic material was originally surmised. In the majority of the implants made in the present study, preendocardial cells were found in positions approximately in correspondence with preepimyocardial cells. Thus it seems reasonable to conclude that the preendocardium is regionally organized at stage 5 in a manner at least roughly similar to the preepimyocardium (Fig. 9). Moreover, most of the implants indicated that the preendocardial regions, like the premyocardial zones, are separated by a median noncardiac area, though not as wide. However, one of the labeled fragments implanted across the midline at the rostral end of the prechordal plate contributed two cells to the endocardium. It is on the basis of these two cells that the prospective endocardium contributing to the conus (region *a*, Fig. 9C) is shown extending to the midline. Since these two cells might have been incorporated into the endocardium in some aberrant fashion, perhaps as a result of microsurgical manipulation of the tissue, their presence hardly seems to justify the conclusion that preendocardium exists in the form of a cardiogenic crescent (Mollier, 1906), as suggested previously (Rosenquist and DeHaan, 1966). On the other hand, the possibility of a median contribution to the endocardium in normal development has not been excluded by these experiments.

Most of the labeled implants made for this study were composed of both mesoderm and endoderm. Each of the recipients harvested and autoradiographed at stage 12 carried a region of labeled mesoderm and one of labeled endoderm, deriving from the same implant. Maps of the

prospective foregut endoderm and the stage 12 tubular foregut, analogous to those in the present work have been constructed and will be presented (Stalsberg and DeHaan, in preparation). From this investigation, however, one point is especially relevant to an understanding of heart formation. If movements of the endoderm from stage 5 to stage 9 are referred to the position of the prechordal plate, all points of the prospective foregut floor are found to move caudally and medially, approaching the midline asymptotically. Such a caudal movement of the endoderm relative to the splanchnic mesoderm was reported earlier (Bellairs, 1953; DeHaan, 1963b; Rosenquist and DeHaan, 1966). The previous interpretation was that the splanchnic mesoderm migrated forward relative to the endoderm, perhaps using the latter as a substratum. The present finding is that among the tissues derived from a single implant, those from the splanchnic mesoderm assume a position intermediate between the more rostrally located somatic layer and the caudally placed endoderm. It is as if the movements of the splanchnic mesoderm were the resultant of relative movements of the layers above and below, one pulling the splanchnic layer forward; the other drawing it backward.

We have recently suggested that the foregut elongates as a result of tension applied at the anterior intestinal portal—tension which arises from the regression of the primitive streak and elongation of the notochord (Stalsberg and DeHaan, 1968). It seems not unreasonable that the same force may be transmitted to the subjacent splanchnic mesoderm, acting to drag that layer medially and posteriorly also. However, such a mechanism, if it applies at all, must be only one among many which bring about the complex movements of the epimyocardial layer described here. Since the free edge of a sheet of tissue is capable of migrating on a substratum in a manner analogous to a cell or cell group (Vaughan and Trinkaus, 1966), the adhesive relations of splanchnic mesoderm and endoderm must continue to be an area of interest.

SUMMARY

Small fragments of mesoderm and endoderm have been transplanted from stage 5 donor embryos labeled with thymidine-^{3}H to corresponding sites in nonlabeled hosts. These fragments heal in and participate in the normal development of the recipient embryo. The distribution of the implanted cells in the tubular heart at stage 12 has been determined by autoradiography, to provide a set of corresponding points in the stage 5 precardiac mesoderm and the epimyocardial and endocardial layers in the stage 12 heart. The tubular heart has been subdivided into 24 de-

fined regions, and the boundaries of those regions have been mapped in the stage 5 mesoderm. The process of heart formation has also been traced through intervening stages by graphic reconstruction and microdissection of the mesoderm layer and by marking the epimyocardial troughs and forming heart tube with particles of iron oxide.

The preepimyocardial mesoderm is organized in the stage 5 embryo in two separate regions, one on either side of the embryonic axis, with a gap of noncardiogenic mesoderm of about 0.8 mm between them. At this stage preendocardial cells are organized into areas generally similar in size and location to the preepimyocardial regions, but they are more widely scattered.

Each lateral heart-forming region can be subdivided into a series of curved bands. The rostromedial band of each heart-forming region forms the truncoconal tissue of the tubular heart. The mid-portion gives rise to the ventricles and the caudolateral area of each region contributes the cells which form the atria.

During cardiogenesis, the preepimyocardial mesoderm behaves as a coherent sheet. It condenses, stretches, folds, and deforms, but it does not lose its integrity as a sheet, nor its continuity with the rest of the layer of splanchnic mesoderm. It does not break up into cells or cell clusters. Endocardial cells, on the other hand, do show some evidence of dispersal as singlets and small groups during formation of the heart tube.

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