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Experimental analysis of closure and reopening of the esophagus in the developing chick

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Iowa State University of Science and Technology
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EXPERIMENTAL ANALYSIS OF CLOSURE AND REOPENING
OF THE ESOPHAGUS IN THE DEVELOPING CHICK

by

Allan LeRoy Allenspach

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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INTRODUCTION

In a previous investigation an account was given of the development and histogenesis of the esophagus of the chick between 5 and 10 days of incubation. The esophagus was subdivided, for the sake of convenience, into three regions which had distinct histogenetic characters: the region of occlusion, just behind the tracheal bifurcation, the vesiculated region, and the reopened region. It was found that the esophagus becomes completely occluded at 5 days (stage 26), and reopening proceeds rapidly at 7½-8 days (stages 33-34). The study was directed toward understanding the possible mechanisms of closure and reopening. To that end, detailed observations were made of histological changes in the organ, and these were correlated with the localization of ribonucleic acid and the enzyme, alkaline phosphatase (Allenspach, '59).

Our previous work suggested that closure of the esophagus was more a result of collapse of the roof to the floor, especially in the region just posterior to the tracheal bifurcation, rather than to a proliferation of cells, as stated in Lillie's Development of the Chick (Hamilton, '52, pp. 384-385). One had the impression that there were actually fewer mitoses in the zone of occlusion than elsewhere. The first part of the present work consists of a series of experiments utilizing the alkaloid, colchicine, a metaphase poison, to cause an
accumulation of mitoses over several hours of time which would
accentuate possible localized differences in mitotic rate.

The results of the above inquiry led to another: if
closure and reopening were independent of mitotic rate, what
other mechanical factors might be operative? Our previous
descriptive work had shown that there was death and desquama-
tion of cells into the lumen, starting at stages 33-34, during
the fusion of vesicles, that the mid-dorsal boundary of the
esophageal epithelium became indistinct, and that cells beyond
this boundary were perpendicularly oriented as though exerting
traction on the dorsal epithelium. Secretory activity was
obviously not a factor in early morphogenesis, since it
appeared after the esophagus was reopened.

If tensions exerted on any part of the epithelium were
contributing to the reopening of the organ, it should be
possible to disturb these by removing calcium from cellular
boundaries and thereby disrupt intercellular connections. The
second part of this study consists of applying Versene to the
embryo by injecting it into the amniotic cavity and permitting
it to act on the esophagus via the pharynx for several hours.
Since Versene aids in the disaggregation of cells (Moscona,
'52; Zwilling, '54), one might expect that the chemical would
aid in the reopening process by making the cells less adhesive
and permitting premature separation of the epithelium, with
subsequent restoration of the lumen. The rationale of this
experiment assumes the correctness of the statement of Townes and Holtfreter ('55, p. 110) that, "in morphogenesis, the forces controlling directed movements must overcome those of cell adhesion."

In summary, the work which follows places the esophagus for the first time in two experimental environments which enable us to determine the role of mitosis in morphogenesis of the organ and give suggestive evidence on the role of cellular tensions during morphogenesis.
REVIEW OF LITERATURE

Colchicine has been used for many years as a tool for biological research. The monograph by Eigsti and Dustin ('55) summarizes the many ways it has been used and the problems which have been attacked. Despite the numerous cytological investigations which have been carried out in an attempt to gain some understanding of the mechanism of colchicine action, the exact method by which it interferes with spindle formation during mitosis is still obscure (Bass, '59, pp. 49-53). Nevertheless, for many purposes, including the present problem, the mere fact that colchicine stops mitosis makes it of considerable use.

The effects of colchicine on chick embryos of various ages have been reported by several investigators. Paff ('39) noted that dilute concentrations of colchicine caused an abnormal accumulation of mitotic figures in the dorsal part of the neural tube of the 48-hour chick and often caused it to remain open. Lallemand ('39) noticed that strophosomy could be produced by application of colchicine to embryos of 40-68 hours, but not on younger or older ones at the same concentration. In embryos of 50-53 hours (stage 14) treated with colchicine, Overton ('58) observed a marked decrease in extent of the area vasculosa associated with an engorgement of blood vessels in the embryo proper. Also, there was a decreased total length of the embryo associated with con-
volutions of the central nervous system and distortion of the notochord, due to lateral bending. Gabriel ('46) was able to produce defects of limb primordia with local applications of colchicine on the chick embryo of 25-28 somites. In any case where there was distortion, the effect was most likely to occur where mitoses were most numerous.

Schultz ('22) studied the mitotic incidence in normal chick embryos ranging from 18-72 hours. In early stages, cellular divisions took place everywhere; later, definite growth centers appeared. Accordingly, development of the early chick embryo was in conformity with the statement of Minot that the rate of growth is diminished wherever differentiation has taken place (Schultz, '22). Derrick ('37) reported that the mitotic indices varied in organs of the chick at earlier stages of development, but dropped progressively with age and differentiation. Richards and Porter ('35) made the same observation in a study of the mitotic index of Fundulus during embryonic development. Hamburger ('48) stated that proliferation proceeded in advance of histogenetic processes and was controlled by special localized structural and physiological conditions.

Colchicine affords us a method of accumulating and counting dividing cells over definite periods of time, thus enabling us to estimate the significance of such divisions in the morphogenesis of various organs and entire embryos.
However, the results may or may not give conclusive answers. Corliss ('53) noted that there are only a few randomly-scattered real differences in mitotic activity in the tissues of rat embryos of 9 days. Apparently, cellular proliferation contributed only to a general increase in the volume of embryonic tissues.

By using a mitotic inhibitor, Bellairs ('54) attempted to determine the role of localized outbursts of mitotic activity in the formation of the foregut of the chick. She concluded that mitoses could not be regarded as a primary factor in closure of the floor of the foregut. Rather, evidence was presented to support the view that a change in cell shapes might be a causal factor in morphogenetic movements and that the high rates of cell divisions might play a part in embryonic induction (Bellairs, '57). It was suggested that the process of increasing the amount of contact between cells in the entodermal epithelium might stimulate an increase in the rate of mitoses, or, alternatively, a higher mitotic rate might lead to an increased contact between cells.

Since the mitotic pattern of the esophagus in the developing chick bears some resemblance to that of the neural tube during early development, a brief discussion of this work is in order. Sauer ('35) regarded the migration of nuclei to and from the lumen of the neural tube with each division to
be most significant, for all dividing cells were observed in the area adjacent to the neural canal. He stated that mitoses were confined to the region of the lumen, not because of any special property of the region, but because the nuclei which were about to divide moved to the region of the lumen to do so. In a successful attempt to show the proliferative capacity of cells in the neural tube of young chick embryos, Watterson et al. ('56) were able to block almost every alar cell in metaphase after 7½ hours of colchicine treatment. Such a demonstration provided evidence that there were no special germinal cells next to the lumen of the neural tube.

Since the introduction of Versene as an effective agent for dissociating cells of chick embryos (Zwilling, '54), literature has accumulated on the effects of Versene on perfused organs (Coman, '54), tissue cultures of chick heart fibroblasts (Dornfeld and Owczarzak, '58) and developing down feathers (Fabiny, '59), as well as isolated mammalian heart (Leitch and Haley, '53). Dornfeld and Owczarzak ('58) observed withdrawal of cellular processes and progressive cellular contraction in nondividing cells of living fibroblasts from the chick heart. In the same study it was reported that Versene did not affect metaphases, but prevented migration of daughter cells, and that new mitoses could be initiated during and after exposure to Versene. With proper concentrations, Versene apparently did not affect viability
or the mean diameter of cells (Weiss, '60).

In an investigation using organ primordia, Fabiny ('59) reported that developing down feathers failed to form from embryonic chick skin of stages 30 to 32 when grown in a culture medium containing 500-525 µg/ml of Versene. Histologically, the feathers showed a thin ectoderm and a diffuse mesodermal pulp. Histochemically, Versene decreased the amount of ribonucleic acid, as shown by staining with toluidine blue, and reduced the activity of alkaline phosphatase by chelating necessary metallic ions (Fabiny, '59).

It is generally presumed that Versene disrupts intercellular connections by removing calcium or other divalent ions from the intercellular matrix. The important role of divalent ions was demonstrated by Gray ('26) with Mytilus tissue where calcium was particularly effective in stabilization of the intercellular matrix. Electron microscope studies of Versenate-perfused liver, a normally tightly adherent tissue, revealed an apparent separation of cells with an eventual loss of integrity of the cell membranes (Coman, '54). From these observations it was inferred that adhesiveness of liver cells depended largely on a molecular orientation at the cell membranes, which was influenced adversely by the removal of the cations. Furthermore, Dornfeld and Owczarzak ('58) observed surface changes in fibroblasts from living chick heart treated with Versene, and interpreted the results
as being due to chelation and removal of divalent ions, notably calcium. DeHaan ('58) reported data on embryos with cardia bifida produced by Versene which suggested that calcium was made unavailable to endodermal and mesodermal cells and the result was disturbance of intercellular relationships and morphogenetic movements. His evidence lent support to the hypothesis that morphogenetic movements are dependent upon proper cellular adhesion and that this adhesiveness is in turn dependent upon calcium bound at cell surfaces.

Leitch and Haley ('53) have shown that the cell membrane is the site of action of Versene. Dornfeld and Owczarzak ('58) concluded that because of the speed with which the effects of Versene could be reversed in cultured cells the action must be limited to the cell surface. These assumptions gain support from Webb and Danielli ('40) who suggested that negatively-charged surfaces acted as reservoirs of divalent ions such as calcium.

The presumption that Versene acts on cell membranes by removing divalent ions from the intercellular matrix has been substantiated by reversibility tests which are quite convincing. Inhibitory effects of Versene on feathers were prevented by addition of Mg or Mn ions (Fabiny, '59). Dornfeld and Owczarzak ('58) found that no cellular responses were provoked by Ca, Mg, or ferrous chelates of Versene. In a
study on the isolated mammalian heart, Leitch and Haley ('53) stated that calcium had a detoxifying effect on Versene.
MATERIALS AND METHODS

White Leghorn eggs were incubated at 38°C for varying periods of time, after a minimum warm-up period of 3 hours. The surface of the shell was sterilized with tincture of iodine, a window was sawed with a thin hacksaw blade, and the pieces of shell removed and discarded. In embryos of stages 26-29 (5-6½ days; Hamburger and Hamilton, '51) injections were made directly into the amniotic cavity. With older embryos it was necessary to drop the chorio-allantoic membrane from the inner shell membrane by piercing the air sac and exerting suction with a pipette bulb. After injection of the experimental solution into the amniotic cavity, the window was sealed with cellophane tape and the egg replaced to the incubator.

For the colchicine* experiments, a stock solution of 1:10^4 was freshly prepared by dissolving 1 mg of the drug in 10 ml Ringer's solution and autoclaving it. Serial dilutions were made with sterile Ringer's solution to give concentrations of 1:10^5 and 1:10^6. Quantities of 0.2 ml of the chosen concentration were injected into the amniotic cavities of chick embryos at stages 26-31. A 1:10^5 solution proved to be the most effective, but other concentrations were also tested. Best results were obtained in short-term

*The colchicine used in these experiments was a crystalline compound obtained from Nutritional Biochemicals Corporation.
experiments of about $2^{1/2}$-12 hours. Only living embryos, as determined by heart beat, were used for histological study.

In using Versene, solutions were freshly prepared before each experiment. Varying concentrations (0.006M-0.01M) were injected into amniotic cavities of embryos at stages 27-34. Since Versene is a chelating agent, it was necessary to dissolve it in Moscona's Ca- and Mg-free solution (Moscona, 1952). It was necessary at the higher concentrations to apply slow heat while agitating the solution in order for the Versene to dissolve completely (Fabiny, 1959). Before application, the Versene solution was sterilized by passing it through a Seitz filter. As before, 0.2 ml was injected into the amniotic cavity. Since reopening proceeds rapidly at stages 33-34 in normal histogenesis of the esophagus, it was necessary to use experimental material younger than this to determine whether or not Versene would actually affect the reopening process. Living, treated embryos were recovered several hours later and washed in Moscona's solution prior to fixing. The commonly-observed ectodermal peeling due to the chemical, Versene, assured us that the treated embryos were affected.

In all experiments the embryos were fixed whole in Bouin's fixative, gradually dehydrated, dealcoholized with benzene, and embedded in paraffin (m.p. 56-58°C) containing 10 percent bayberry wax, and stored in the refrigerator until
Sections were cut at 7μ and mounted on albumenized slides. All material was stained with iron-hematoxylin* after which the sections were cleared in xylene and mounted with Hartman-Leddon mounting medium.

*Coleman and Bell Co., certification number FH-15.
RESULTS

Mitotic Patterns during Closure and Reopening of the Esophagus

The results to be reported here are based mainly on embryos which were treated with a 1:10^5 colchicine solution (0.002 mg colchicine/0.2 ml saline) for 2½-12 hours. This was the most effective concentration in that it acted upon the esophageal epithelium and still permitted survival of the embryo for the requisite periods of time. Even so, some selection of embryos was necessary because many embryos died too soon or showed no inhibition of mitoses in the internal tissues. No doubt this variation was due in large part to the method of applying the colchicine. Dosages approaching the concentration of 1:10^4 were usually lethal to embryos of 5-7 days after several hours of exposure.

For convenience, results of the study of the mitotic patterns have been set down according to four regions along the length of the esophagus (Fig. 12). These are regions of distinctly different morphology, as described in our previous study (Allenspach, '59). It will be seen that the zone of mitotic activity differs in the various regions and changes during development.
Region of the tracheal bifurcation

Generally, mitotic figures are located in close proximity to the basement membrane during the period of closure. During normal development very few mitoses are observed in the roof and floor epithelia (Fig. 1). After treatment with colchicine it is noted that mitoses are not localized at the juncture of roof and floor epithelia, but rather are scattered both ahead and behind the point of fusion (Fig. 2). They appear more frequently in the floor than in the roof. It would be difficult to believe that mitoses are frequent enough to account for the sudden closure of the esophagus, since on occasion the occlusion may occur within the space of three sections (21μ). Further, the fact that there are cytoplasmic extensions of the epithelium into the lumen suggests that closure is due to adhesion of epithelial cells (Fig. 2).

Region of occlusion

After treatment with colchicine, it is noted that mitoses are scattered randomly in the occluded esophagus. Some mitoses appear near the basement membrane, while others occur in the center of the solid epithelial bar (Fig. 4). Fig. 5 shows a typical pattern of colchicine inhibition of dividing cells in the narrow, bifid esophagus. Notice that dividing cells are located both at the basement membrane and in the loosely-organized epithelium. The mesenchyme contains many
dividing cells.

Region of vesiculation

It was reported previously that the vesiculated region first appeared at stage 28 (Allenspach, '59). In rare cases it has been seen in stage 27. With the appearance of vesicles, there is an abrupt increase in mitotic rate. Fig. 6 is a transection of the normal esophagus in the vesiculated region. After short periods of treatment with colchicine (4-5 hours), many mitotic figures accumulate in the epithelium, particularly around the vesicles. Mitotic counts of alternate serial sections reveal an apparent crest in activity at about stages 29-30, with a decline at stage 31. With epithelial differentiation there are fewer mitoses, and these are located near the lumen (Fig. 9).

Although the impression may have been given that vesicles and dividing cells go hand in hand, as shown in Fig. 8, this is not always the case. On the contrary, there may be dividing cells at some distance from the vesicles (Fig. 7). Thus, the mitoses are not necessarily causally related to vesiculation.

Open region

As is observed during normal histogenesis, the dividing cells are almost always next to the lumen (Fig. 10). Very
infrequently are mitotic cells observed near the basement membrane. A similar situation obtains upon treatment with colchicine (Fig. 11). Dividing cells almost always withdraw to the edge of the lumen, becoming vesicular in shape (Fig. 11).

Colchicine effected only a slight increase in mitotic figures as determined by mitotic counts in alternate sections of portions of the open region. Attempts to accumulate many layers of metaphase plates around the lumen by the use of progressively longer exposures to colchicine failed. Usually metaphase plates will collect in only one, or possibly two, layers around the single lumen. In this respect the esophagus differs from the neural tube, as described by Watterson et al. ('56).

Closure and Reopening of the Esophagus in the Presence of Versene

Hosts ranging in age from 5-5½ days (stage 27) to 8 days (stage 34) were used in these experiments. Concentrations of 0.006M to 0.01M Versene gave the most satisfactory results.

Region of the tracheal bifurcation

Embryos of stages 27 and 28 occasionally show separation of the epithelium at the roof-floor juncture. Embryos of stages 29 to 34 apparently are not affected in this region by
the exposure to Versene. Seldom are aggregates of cells observed lying freely in the lumen.

Region of occlusion

Separation of the epithelium occurs primarily at the median line and is usually found in only short regions of the occluded bar of the esophagus (Fig. 12). The first indications of epithelial separation occur at about stage 29 (Fig. 14) and can be observed in the occluded region through stage 32 (Fig. 12). Sagittal sections of the esophagus reveal an interesting dilatation, corresponding with the epithelial separation and possibly a consequence of it (Fig. 12).

Oil immersion observations show that there is not always complete separation of the cytoplasmic fibrils. However, many intercellular connections are interrupted, suggesting a disaggregation of epithelial cells.

In contrast to observations in the region of the tracheal bifurcation, the basement membrane of the epithelium at the dorsal mid-line of the occluded zone is not smooth and conspicuous. Rather, the membrane is correspondingly indistinct in regions where there is separation, whereas to either side it is distinct and continuous (Fig. 14). In most cases the mesenchymal cells adjacent to the basement membrane of the epithelium are oriented perpendicular to its surface.
Region of vesiculation

It is in this region that the most significant results can be observed. During normal histogenesis the membranes limiting the vesicles are continuous. However, in Versene-treated embryos the vesicular-limiting membranes display a tear or rupture in almost every case. Abnormal separation of the roof epithelium from the floor epithelium is first detected as early as stage 29, much earlier than observed in normal histogenesis, and is repeatedly observed in treated embryos through stage 34, the stage at which normal reopening occurs. As stated in a previous investigation (Allenspach, '59), the normal reopening of the esophagus with accompanying separation and desquamation of epithelium proceeds rapidly at stages 33-34.

Stages 30 and 31 show some interruption of the cellular bridge at the median line between the major esophageal vesicles. Fig. 16 shows a typical and striking result of Versene treatment in an embryo at stage 32. Interruption of the intercellular connections is accentuated between vesicles, particularly at the level of bilateral vesicles with inter-spaced smaller vesicles. Above the still-unopened cellular bridge with its Versene-induced cleft is the very indistinct dorsal basement membrane (Fig. 16). The correlation of these two conditions has been noted previously.

Sagittal sections reveal that there is an obvious
separation of the solid portion of the esophagus into roof and floor after treatment with Versene, which is most obvious in the posterior portion of the vesiculated region and proceeds cephalically (Fig. 13). Cytoplasmic intercellular connections are affected as shown by the fact that the vesicular membranes are disjoined (Fig. 17), rather than continuous (Fig. 6). Observations under higher magnification reveal loose, jagged, cytoplasmic projections in the lumen, and a dividing cell with a torn polar cap, apparently due in part to the tensions existing in the system (Fig. 18).

At stages 33-34 there is also a separation of the esophageal epithelium, due in part to the presence of Versene. Separation of the epithelial cells between the vesicles has resulted in the tearing of cell membranes (Fig. 15). Occasionally denuded nuclei will appear adjacent to the lumen.

Open region

In all of the cases studied, which include stages 27-34, there apparently is no effect on the epithelium in the open region of the esophagus.

There was no noticeable effect of Versene on dividing cells except secondarily through tearing, as illustrated in Fig. 18.
DISCUSSION

With the setting up of two different experimental environments some light has been shed on the mechanisms existing during closure and reopening of the esophagus. Exposure of the esophagus via the oral cavity to dilute concentrations of colchicine in the amniotic cavity leads to accumulations of mitotic figures, which enable us to evaluate the role of cell proliferation on closure and reopening; and, exposure of the esophagus in a similar manner to Versene solutions gives evidence on the possible influences of cell adhesions and morphogenetic tensions on these processes in the esophageal epithelium.

Apparently cell division is not involved in either the closure or reopening. Lillie ('19) stated that the esophagus became occluded by a proliferation of epithelial cells. However, in a previous investigation (Allenspach, '59), mitoses were seen to be very infrequent in the region of the tracheal bifurcation during closure. If cell proliferation were an important mechanism in closure one would expect to find dividing cells in abundance at the roof-floor juncture in the posterior pharyngeal region and the occluded region. This was not the case. Colchicine-inhibited cells were found to be scattered widely along the broad esophageal bar, both ahead and behind the point of fusion, but not particularly localized at the roof-floor juncture (Allenspach and Hamilton, '60).
Nor were there many dividing cells in the completely occluded region of the esophagus. It seems that the incidence of dividing cells in these regions is not sufficiently high to merit consideration as a primary factor in the closing process. Bellairs ('54), using a mitotic poison to determine the role of cell division on the formation of the floor of the foregut in the chick, came to the same conclusion.

Instead, occlusion of the esophagus seems to be effected by a collapse of the roof of the pharynx to the floor with subsequent fusion of the epithelium. The fact that closure of the esophagus is accompanied by a change from a sharply-defined inner limiting membrane to an irregular boundary with extending protoplasmic processes suggests that closure is due to cellular adhesions.

Hamburger ('48) has stated that the rate of mitotic activity in any one region is very likely governed by special structural and physiological conditions which prevail in that region. Apparently the conditions are favorable in the vesiculated region, for with the appearance of vesicles there is an abrupt increase in mitotic rate. Dividing cells are almost always located adjacent to the vesicles. However, the appearance of the vesicles cannot be ascribed entirely, if at all, to the dividing cells, for it is not uncommon to see vesicles with no dividing cells in the adjacent epithelium. Thus, mitoses are not the primary cause of vesiculation and
the reopening process.

The fact that there are dividing cells next to the vesicles must be a secondary phenomenon, due to a withdrawal of the dividing cells to their attachment site when rounding up for division. This phenomenon was described by Sauer ('35) in a study of mitosis in the neural tube of the chick. He conceived of a migration of nuclei to and from the lumen at each division, the migration to the lumen being evidently associated with the fact that cells assume a rounded form in mitosis and that the cells had their bases attached to the inner limiting basement membrane.

It is very unlikely that secretion is responsible for the appearance of vesicles, since the first vesicles that appear, particularly at the anterior end of the esophagus and in the early stages, contain no trace of secretion. The secretion appears later, after much merging of vesicles has already occurred (Allenspach, '59). The fact that cell death causes vesiculation and the reopening process, at least at older stages, has been observed. The significance of cell degeneration in the reopening process will be discussed later.

In the completely reopened esophagus, as in the vesiculated region, dividing cells are consistently restricted to the area adjacent to the lumen. Apparently there is a limit to the proliferative capacity of epithelial cells in this region, for blocking of layers of metaphase cells was not evident in
our study. Only two layers of dividing cells at most were seen after adequate exposure to colchicine. These results are in contrast to results obtained by Watterson et al. (1956), who were able to demonstrate by the colchicine technique that almost every cell in the alar plate of the neural tube had proliferative capacity, i.e., almost every cell in the alar plate was blocked at metaphase after 7½ hours of treatment with colchicine.

During the period of normal development between stages 26 and 33 there is a rapid differentiation and growth accompanied by mitotic activity in the epithelial cells, particularly in the region of vesiculation (Allenspach, 1959). Analysis of the colchicine-treated embryos shows a particularly high mitotic rate in the epithelium surrounding the vesicles. Comparing the mitotic picture of the occluded region (Fig. 5) with that of the vesiculated region (Figs. 7 and 8), it is obvious that the latter is a more actively growing region. Analysis of the colchicine-treated material also suggests that there is a peak in the mitotic activity of the epithelium at about stages 29-30, the activity diminishing as differentiation proceeds. Thus it appears that the vesiculated region may be responsible for increasing the volume of the tissue. Corliss (1953) reported a progressive, over-all increase in the mitotic activity of the rat embryo at 9 days. He considered that this cellular proliferation contributed only to
a general increase in the volume of embryonic tissue. In the chick, it seems probable that the high mitotic activity in the vesiculated and reopened regions is concerned with the lengthening of the esophageal tube as the neck of the embryo rapidly elongates.

While we have already mentioned that cellular proliferation was apparently not a primary factor in the closing process, the results obtained with Versene fail to give us positive evidence on the nature of the closing mechanism. Apparently cell adhesion is not the only factor involved in the closing process. The presence of Versene in the amniotic fluid did not prevent occlusion of the esophagus. Apparently any decrease in adhesiveness caused by the Versene was not significant enough to affect fusion of the epithelium in the region of the tracheal bifurcation.

Since an upset of intercellular relationships and morphogenetic movements by removal of calcium ions with Versene has been demonstrated by other workers, it would not be unreasonable to expect some disturbance in the esophageal epithelium during the process of reopening. This is exactly what happens.

The first signs of a weakness of intercellular relationships and separation of the epithelium in the occluded region are observed at stage 29 (Fig. 14). Fig. 12 is a typical example of the effect of Versene along the length of the esophagus. Such disturbances are regularly seen in treated
embryos up until the time of normal reopening (stages 33-34). The separation of epithelium in the occluded region in Fig. 12 is accompanied by a corresponding dilatation which suggests that there may be a traction exerted on the dorsal epithelium at that point by adjoining cells or tissues.

The separation of the epithelium in the presence of Versene is most accentuated in the region of vesiculation, at the level of the bilateral primary vesicles (Fig. 16), and proceeds from this point forward. Normally, restoration of the lumen is accompanied by cell death and desquamation at stages 33-34. In the presence of Versene the epithelium separates as early as stage 29. It must be mentioned, and this is significant, that no necrosis is observed during, or as a result of, the premature separation of the epithelium in the vesiculated region (Fig. 17). Apparently the Versene has effectively removed the interstitial calcium which has in turn made the cells less adhesive, allowing cells to disperse according to the tensions placed upon them. The experiments tend to support the hypothesis that morphogenetic movements are dependent on proper cell adhesions, and that cell adhesiveness is in turn dependent upon intercellular calcium (DeHaan, '58, p. 367). Evidently there are tensions existing in the surrounding tissues which are actively assisting in the reopening process as early as stage 29 but which are not expressed normally until stages 33-34, at which time cell
death permits separation of the roof from the floor to restore the lumen.

It was observed that there was no necrosis during premature epithelial separation. Apparently cell death plays a significant role in the reopening process of the esophagus. Glücksmann ('51, p. 82), in an admirable survey on cell degenerations in relation to integrations of tissues, states that "while some degenerations have no obvious function in embryonic development, others seem to play a significant role in embryonic processes, e.g., the morphogenesis and histogenesis of tissues and organs." Cell deaths are related to the formation of the lumen in completely or partly occluded glands and portions of the intestinal tract (Glücksmann, '51). Cell death and desquamation are obvious during the reopening process of the esophagus at about stages 33-34. The fact that there are no cell deaths with premature separation suggests the possibility of a timing mechanism for cell death in the organ. Such localized deaths might lead to the formation of vesicles, followed by complete separation.

The fact that tensions are most obvious in the posterior part of the vesiculated region after Versene treatment (Fig. 13) may be correlated with the fact that normal restoration of the lumen proceeds in a caudal-cephalic sequence, beginning at the posterior level of the vesiculated region (Allenspach, '59).

There is no obvious effect of Versene on the epithelium
of the reopened esophagus.

Lack of a sharp dorsal boundary to the esophageal epithelium and the vertical orientation of the cells led us to suspect a migration of cells between the epithelium and mesenchyme. It was also observed that the esophageal epithelium reorganized from the haphazard form of the occluded region to the stratified type in the definitive organ, the initiation taking place around the most lateral vesicles and proceeding towards the midline as the vesicles enlarged (Allenspach, '59). The results of the Versene experiments lead us to an entirely different interpretation of these phenomena. Rather than a migration of cells between germ layers, the radial orientation of the cells and the indistinct basement membrane may be an expression of forces pulling on the epithelium. Wherever there are apparent forces the basement membrane is indistinct and the adjacent epithelium is less well arranged. Thus, the lateral stratified epithelium is associated with a distinct basement membrane, and the unorganized medial epithelium and indistinct dorsal basement membrane of Fig. 16 are inter-related. The fact that the epithelial cells intergrade with the mesenchymal cells at the dorsal mid-line reflects the increased contact of surfaces between the two types of cells during the important process of reopening (Fig. 15). Their radial, elongate shape and slender attachment to the epithelium suggest that these cells are
trying to move dorsally and thus are exerting traction on the epithelium. When the lumen is restored and the tension is relieved, the basement membrane returns to its normal position, and the epithelium acquires an orderly arrangement.

Other possible explanations for the separation of the epithelium cannot be disregarded. First of all, we can dismiss the possibility that this may be fixation artifact, for fixation was excellent in all other tissues examined in the treated embryos. Such crevices never appeared in the fixation and processing of normal embryos. Secondly, although there is no evidence against the possibility that the cells have undergone progressive contraction during treatment with Versene (Dornfeld and Owczarzak, '58), the evidence suggests that Versene functions primarily to loosen intercellular substances and permit tractions to be expressed. Such a statement is based on the fact that the epithelial separation along the length of the esophagus is much too restricted to be due to cellular contraction. Thirdly, it is not likely that the disaggregation that occurs with 0.006M and 0.01M Versene is due to an osmotic imbalance. DeHaan ('58, p. 364) stated that concentrations of 5-6mM did not cause osmotic imbalance to stage 10-12 chick embryos. The concentrations used in these experiments were just above the limits used by DeHaan ('58, p. 364) to produce cardia-bifida in early chick embryos. Also, the concentrations of Versene were just severe enough to
cause a peeling of the ectoderm from the embryo, but not enough to cause total disaggregation of the tissues. Lastly, in view of the fact that Versene treatment may lead to loss of integrity of the cell membranes (Coman, '54), disintegration of the cell membrane cannot be completely ruled out. It is not likely that cell membranes were broken down by Versene, however, since concentrations used in these experiments were much lower than those used by Coman. Any disturbances of cell membranes, as observed in the dividing cell on Fig. 18, may be attributed ultimately to tension effects exerted on the epithelial cells. Obviously, in this case, rupture of the dividing cell did not occur before fixation, else the mitotic process itself would have been disrupted. Fixation probably accentuated the built-in tensions of the living tissue, already weakened by Versene, thus tearing off the polar cap of this particular cell.
SUMMARY AND CONCLUSIONS

1. Closure of the esophagus is accompanied by a change from a sharply-defined, inner, limiting membrane to an irregular boundary with extending protoplasmic processes which give it an adhesive appearance.

2. Apparently cellular proliferation is not involved in the closing process. Mitoses are very infrequent in the region of the tracheal bifurcation during closure.

3. With the appearance of vesicles during the process of reopening, there is an abrupt increase in mitotic rate. Dividing cells blocked by colchicine are almost always located adjacent to the vesicles.

4. The appearance of dividing cells adjacent to the vesicles is presumably a secondary phenomenon, due to withdrawal to their site of attachment and rounding up for division.

5. It appears that the high mitotic activity in the vesiculated region is responsible primarily for increasing the volume of the tissue during elongation of the neck. The peak of mitotic activity appears to be at about stages 29-30.

6. In the completely-reopened esophagus, the occurrence of mitoses adjacent to the lumen resembles the condition in the "germinal zone" of the chick neural tube.

7. In the presence of Versene there are signs of premature splitting of the epithelium in the occluded region at
stage 29. Separation of the epithelium is accompanied by a corresponding dilatation of the organ. The separation is most accentuated at the posterior portion of the vesiculated region, and proceeds in a caudal-cephalic direction.

8. Apparently, Versene has removed the intercellular calcium, which has in turn made the cells less adhesive, allowing tractions on the esophageal epithelium to express themselves prematurely.

9. The orientation of the cells at the indistinct dorsal epithelial boundary may be an expression of forces exerted on the esophageal epithelium. Apparently the indistinct dorsal boundary and a lack of orderly epithelial arrangement are inter-related phenomena during the reopening process.

10. Normally, cell death is requisite for reopening. Premature separation of the esophagus in the presence of Versene is not accompanied by cell death. This suggests that cellular adhesions must be overcome by existing forces to effect reopening of the occluded organ.


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Explanation of Figures

1. Cross section through the tracheal bifurcation (stage 27) showing the closing process. There are very few dividing cells in the epithelium. Stained with toluidine blue. (From previous work for the M. S. degree). X130.

2. Cross section through the posterior pharynx in the region of the tracheal bifurcation in an embryo (stage 27) treated with a $1 \times 10^5$ colchicine solution for 8 hours. Note the paucity of dividing cells in the epithelium, sparsely scattered in both roof and floor. Also, observe the projecting cytoplasmic processes which give the epithelium an adhesive appearance. Iron-hematoxylin. X300.

3. Cross section through the normal, broad, occluded esophagus just posterior to the tracheal bifurcation (stage 29). There are very few dividing cells in the epithelium. Iron-hematoxylin. X220.

4. Cross section through the broad, occluded esophagus just posterior to the tracheal bifurcation in an embryo (stage 29) treated with a $1 \times 10^5$ colchicine solution for 4 hours. Note the widely scattered accumulation of mitotic figures in the epithelium, primarily in proximity to the basement membrane. Iron-hematoxylin. X220.
Explanation of Figures

5. Cross section of the occluded region in an embryo (stage 29) treated with a 1:10^5 colchicine solution for 4 hours. Mitotic figures are located mainly at the basement membrane, but are found occasionally in the central, unorganized epithelium. Iron-hematoxylin. X480.

6. Cross section through the vesiculated region in a normal embryo (stage 29), showing mitotic figures localized around the vesicles. Notice the indistinct dorsal boundary of the epithelium. Iron-hematoxylin. X500.

7. Cross section through the vesiculated region in an embryo (stage 27) treated with a 1:10^5 colchicine solution for 5 hours. Notice the accumulation of dividing cells in the epithelium, primarily adjacent to the vesicles. Only an occasional cell division occurs in the peripheral epithelium. Iron-hematoxylin. X500.

8. Cross section through the vesiculated region in an embryo (stage 29) treated with a 1:10^5 colchicine solution for 4 hours. Mitotic figures have rounded up, withdrawing to the epithelium adjacent to the vesicles. Iron-hematoxylin. X480.

9. Cross section through the vesiculated region in an embryo (stage 33) treated with a 1:10^5 colchicine solution for 12 hours. Dividing cells, although few in number, are localized in the epithelium adjacent to the vesicles. Iron-hematoxylin. X300.
PLATE 3

Explanation of Figures

10. Cross section through the reopened region of a normal embryo (stage 28) showing the typical radial arrangement of the epithelium with dividing cells localized next to the lumen. Iron-hematoxylin. X500.

11. Cross section through the reopened region in an embryo (stage 29) treated with a $1:10^5$ colchicine solution for 4 hours. Observe the accumulation of vesicular, dividing cells adjacent to the lumen, resembling the "germinal zone" of the chick neural tube. Iron-hematoxylin. X480.

12. Sagittal section showing the various regions of the esophagus (stage 32). From left to right: posterior pharynx; occluded region; region of vesiculation. The posterior reopened region is not shown. In the presence of 0.01M Versene for 12 hours, the esophagus exhibits a separation of the epithelium in the occluded region, with a corresponding dilatation of the organ (arrow). Iron-hematoxylin. X125.

13. Sagittal section through the vesiculated region (stage 32) after treatment with 0.01M Versene for 12 hours. Note the obvious separation of the roof from the floor. The results are accentuated in the posterior portions of the vesiculated region. Note the mass of cells lying freely in the lumen. Iron-hematoxylin. X400.

14. Cross section of the esophagus (stage 29) showing a separation of the epithelium of the roof from the floor after treatment with 0.01M Versene for 10 hours. Normally, the occluded region appears as shown in Fig. 5. Iron-hematoxylin. X440.
PLATE 4

Explanation of Figures

15. Cross section through the vesiculated region (stage 34) after treatment with 0.006M Versene for 11 hours. There is the regular appearance of vesicles during the reopening process; however, observe the unnatural separation of the epithelium on the right side of the esophageal bar (arrow). The dorsal epithelium lacks a sharp basement membrane. Iron-hematoxylin. X320.

16. Cross section through the vesiculated region (stage 32) after treatment with 0.006M Versene for 10 hours. Intercellular relationships are interrupted in the epithelial bridge between the bilateral vesicles. (Compare with Fig. 9). The epithelium lacks a distinct basement membrane dorsally. Iron-hematoxylin. X400.

17. Cross section through the vesiculated region of the esophagus (stage 32) after treatment with 0.006M Versene for 10 hours. Notice the interruption of intercellular connections and the torn vesicle-limiting membranes (arrow) in the epithelial bridge between the vesicles. Iron-hematoxylin. X760. v = vesicle.

18. A higher magnification of Fig. 17, showing separation of the epithelium between vesicles after treatment with Versene. Note the freely-extending protoplasmic processes and the interrupted vesicle-limiting membrane. The metaphase cell displays a torn polar cap moving away with the dorsal epithelium (arrow). Iron-hematoxylin. X1700.