

STUDIES ON MORPHOGENESIS IN EARLY AMPHIBIAN EMBRYOS

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THE REASONS WHY SECONDARY INDUCTIONS ARISE ON EMBRYOS OF XENOPUS LAEVIS
CENTRIFUGED AT THE LATE BLASTULA OR EARLY GASTRULA STAGES.

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INTRODUCTION

The study of the effects of centrifugation on the development of amphibian embryos dates back to the early years of this century. The earlier experimenters worked with the unsegmented and newly cleaved egg (O. Hertwig 1904; Konopacka 1908; Jenkinson 1914). Jenkinson described many cases of abnormal development following centrifugation before cleavage. Some embryos showed only trivial irregularities in the formation of axial structures. The notochord was progressively reduced to the point of absence in embryos more severely affected, these showed also a parallel reduction of the nervous system. The most seriously affected embryos had no trace of a neural plate. The sense organs were ill-formed or absent and Jenkinson concluded that their development is particularly susceptible to centrifugation.

Only defect anomalies arise in embryos centrifuged before the mid to late blastula stage; but superimposed on these, a spectrum of accessory abnormalities appear on Anuran embryos between the mid blastula and early gastrula stages. These were first described by Banta and Gortner (1915) and later by Reverberi and Pasquini (1929). Their descriptions agree in all essentials with those of later workers, Beans, Risley, and King (1934), Sehechtman (1937), Karasaki and Yamada (1955), Pasteels (1953a, 1953b, 1954). Pasteels has provided the most important references in this field; his work has a unified presentation in three papers in the Journal of Experimental Embryology and Morphology. These authors describe small finger-like secondary tails arising on the ventral surface of the tadpole, containing spinal cord, notochord, and somite mesoderm; only in one or two species does centrifugation produce larger accessory abnormalities. The development of *Xenopus laevis* is more readily diverted in this way than any other species so far tested, and hence is a specially suitable species to use in an attempt to discover the reasons why accessory abnormalities occur. Such a study might shed light upon the processes of embryonic induction and individuation. In the course of this work many embryos came to hand bearing far-reaching accessory abnormalities of types

hitherto undescribed. These will be described here only in sufficient detail for the main purpose of this thesis, which is an investigation into causes.

Pasteels and others have used the term "secondary induction" in preference to the better, purely descriptive term "accessory abnormality". "Secondary induction" is so well established that it has been adhered to in this thesis. It should be remembered that it is used in a purely descriptive sense, and has no causal implications: Pasteels believes that secondary inductions arise without induction.

In the course of this study it will be necessary to refer to the background facts. Here is a summary mainly taken from Pasteels. Many of the points do not apply to *Xenopus* without qualification, but they form a valuable body of generalizations.

1. Secondary inductions have arisen on all Anuran species so far tested; *Pleurodeles waltii* and *Ambystoma punctatum* are the only Irodele species that have given inductions. Compared with Anurans they are small, simple, difficult to produce and occur in a low frequency.

2. Inductions arise on embryos centrifuged between the mid blastula and early gastrula stages. The ease with which they are produced increases slowly from the beginning of the sensitive period, is greatest just before the dorsal lip appears, and decreases rapidly after the onset of gastrulation.

3. The inductions obtained, after treating early in the sensitive period, are qualitatively different in some species from those produced after treating late. This does not apply to *Xenopus*.

4. The frequency of inductions increases with the centrifugal force applied and the period of application; the overall size of the inductions may increase, but their qualitative nature is not changed, it depends solely on the age of the embryos.

5. The immediate effect of the treatment: the blastocoele is obliterated by a centrifugal displacement of the blastocoele roof, the blastocoele fluid is forced out of the embryo and collects in the perivitelline space. The blastocoele roof lies on the main yolk mass, a contact between these two layers seems to be essential if an induction is to arise.

6. The secondary inductions arise from a portion of the collapsed blastocoele roof.

7. Inductions develop from ectoderm. Mesoderm may be incorporated into the secondary axis but this is a regulative phenomenon, and follows the "autonomous" differentiation of ectoderm.

8. The secondary nerve cord lacks a prosencephalon.

Theories advanced to explain why secondary inductions arise after centrifugation.

Bagini (1925) considered the ectoderm to be already determined in the early gastrula; she envisaged a reshuffling of ectodermal anlagen. This is of only historical interest.

Pasquini and Reverberi supposed that the distribution of mesoderm during gastrulation may be irregular in embryos distorted by centrifugation.

Beams, Risley, and King, without coming to any very definite conclusion themselves, were the first authors to distinguish between two hypotheses later to find adherents in Schechtman and Pasteels - the anomalies may have an intra- or an extracellular origin. In the first case, according to Beams, Risley, and King, centrifugation may cause "the displacement of mitotic figures, the injury and killing of certain cells, or an intensive stratification of materials", capable of altering the morphogenetic capacities of individual cells. Secondly, centrifugation may change "the composition or organisation of the inductors or the organising centres" at an extracellular level "resulting in a disturbance of the normal inductive processes". Schechtman favours the extracellular hypothesis, so do Karasaki and Yamada, and Saxen and Toivonen (1955). Pasteels' ideas are based firmly on the intracellular hypothesis.

Schechtman, in his short paper, distinguishes carefully between defect and accessory anomalies. He finds certain defects, microcephaly for example, correlated with the amplitude of the secondary structures. Defect anomalies can be studied in their purity on species which do not give secondary induction (see Pasquini 1942). Schechtman considers that the defect anomalies may be due to various causes, including those mentioned above. The inductions he suggests are due to contact between ectoderm and mesoderm brought about by the collapse of the blastocoele roof. "Possibly induction occurs by this contact, or portions of the mesoderm may remain adherent to the ectoderm";

if the last point is true, mesoderm will be carried on to the ventral surface of the neurula.

Using *Hyla regilla* eggs he obtained secondary inductions identical with those produced by centrifugation, by two quite different means, but both carried out during the stages sensitive to centrifugation.

i. By implanting portions from the ventral and lateral blastoporal lips into the blastocoele.

ii. By attempting to distort the embryo in the same way as does centrifugation, by pressing the blastocoele roof downwards on to the yolk mass. However, Schechtman does not state the number of successful inductions he was able to produce in this way nor the proportion. This is the only experimental evidence he brings forward to support his suggestion above; it is of interest in connection with Pasteels' theory.

In framing his own theory to account for the effects of centrifugation, Pasteels leans heavily on the results of two series of explantation experiments, ~~continued above~~

In the first experiment he explanted the animal hemisphere ectoderm from embryos of *Rana temporaria*, immediately after centrifugation. There were thirty-two explants in this series, taken from late blastulae due to gastrulate six hours later. Twenty-nine of these survived, seventeen developed a nerve cord, and sixteen of these contained notochord. In another experiment thirty-two explants gave thirty-one nerve cords and notochords, twenty-one of these bore somites. Pasteels does not give the proportion of secondary inductions arising in intact, centrifuged embryos from the same batches. This figure should be closely correlated with the fraction of explants containing nerve cord, before the same cause can be ascribed to both. Pasteels describes the appearance of these explants as follows: "Le tissue neural reste compacte, et s'il y dessine une lumière, celle-ci sera tortueuse et de calibre irrégulière; en aucun cas on ne peut reconnaître un rhombencéphale, ni une moelle, ni quelque'autre organe neural. La corde ne présente aucune polarité; elle forme soit de gros amas soit de boudins tortueux. Dans ces masses chordales, les cellules les plus vacuolisées trouvent souvent à la

peripherie, tandis que les cellules les moines agées, cytologiquement, reste au centre du nodule. Il est evident que dans ce cas on ne peut parler ni de tête, ni de tronc, ni de queue."

In the second series of experiments portions of ectoderm taken from embryos immediately after centrifugation were grafted on to untreated embryos; the host species, its stage at the time of grafting, and the position of the graft were varied. These experiments confirmed the result from the first series, that the ectoderm had acquired an autonomous faculty for differentiation after centrifugation. In one particular instance it proved to be a more sensitive means of revealing a capacity for self-differentiation in centrifuged ectoderm: early blastulae of *Rana temporaria* are relatively insensitive to centrifugation compared with the early gastrula, the proportion of inductions for a given treatment being very much lower in the former. Ectoderm explanted from early blastulae immediately after centrifugation develops into epidermis only, whereas portions of ectoderm grafted near the primary hind brain of a neurula develop into neural tissue.

Pasteels believes that the secondary inductions are due to autonomous differentiating tendencies arising in centrifuged ectoderm by virtue of the stratification of the cell contents. Thus, in contrast with Schechtman, Pasteels forms his hypothesis on the first alternative of Beams, Risley, and King, namely the intra-cellular hypothesis. He considers the results of his experiments to rule out the possibility of an inductive stimulus passing from one tissue to another. The transfer would have to be very rapid - too rapid to be seriously considered at the time Pasteels wrote.

Yamada (personal communication) has repeated Pasteels' explantation experiment using *Bufo vulgaris* embryos, and confirms Pasteels' result.

Karasaki and Yamada (1955) have reported the results of experiments designed to test Pasteels' theory. They made ectodermal explants from two species of Anuran, using stages within the period sensitive to centrifugation. The explants were allowed to heal and then centrifuged at a level sufficient to produce secondary inductions in

intact embryos. They used late blastulae and early gastrulae of *Rana japonica* and *Bufo vulgaris*; not one explant out of a total of eighty-five developed neural or mesodermal structures; of two hundred and forty-five control embryos receiving the same treatment, ninety gave secondary tails, forty-eight head malformations and only forty-seven were normal.

Further, they wished to test Pasteels' suggestion that the effect of centrifugation on ectoderm is similar to the "sub-lethal" cytolysees that Holtfreter demonstrated in newts. At that time the only demonstration of sub-lethal cytolytic neuralizations came from work with *Urodela* species, all of which are insensitive to centrifugation. It was not certain that Amuran ectoderm would display the same properties. Moreover, inductions produced by sub-lethal cytolysis in *Triturus* were all archencephalic, whereas those produced by centrifugation are rhombencephalic. Explants of ectoderm from the early gastrulae of *Rana nigromaculata* and *Rana pipiens* were exposed to alkaline solutions, they formed neural tissue and mesenchyme in good proportion. But, as in the case of *Triturus*, the neural tissue was archencephalic in every case where it achieved sufficient organisation to permit precise identification.

Karasaki and Yamada conclude from these results that the effect of centrifugation on ectoderm is dissimilar to the sub-lethal cytolysees of Holtfreter. They go further and consider that the treatment has no direct effect upon the ectoderm at all. They qualify this: "the possibilities are not excluded that the centrifugal force affects the ectoderm directly when it is within the whole intact embryo, perhaps by changing its relationships with other components of the embryo".

In the work to be described it is shown that two causes interact to give the varied spectrum of abnormalities seen in *Xenopus* embryos after centrifugation. It will be shown that the small secondary axes growing from the posterior ventral surface of otherwise normal embryos are due to an induction in presumptive belly epidermis.

Stronger centrifugation causes these inductions to include anterior areas of the ventral surface. At the higher levels of treatment a second effect becomes super-

imposed on the simple mechanism, namely an abnormal distribution of mesoderm during gastrulation. The evidence for the former cause is mainly experimental, the latter mainly inferred from a retrogressive analysis of centrifuged embryos.

MATERIALS AND METHODS

Xenopus embryos obtained by injecting toads with chorionic gonadotrophin. When necessary the eggs were decapsulated chemically. Holtfreter solutions prepared without sodium bicarbonate buffered according to Deuchar (1951), and c. 5000 i.u. penicillin and streptomycin added per litre after autoclaving. Embryos and explants cultured at 18°C except where otherwise stated. An MSE electric centrifuge was used throughout, the time of treatment is measured from the time the centrifuge was set in motion to the time the motor was switched off or disengaged.

A DESCRIPTION OF THE LATE BLASTULA AND EARLY GASTRULA STAGES OF XENOPUS

The late blastula and early gastrula stages of *Xenopus* have been described by Nieuwkoop, see Normal Table, and Nieuwkoop and Florschütz 1950. This author, working from sections, has described the location and movement of the various layers, the distribution of pigment and the surface coat. Despite this the early embryology of *Xenopus* is poorly known, and these two stages were examined here for two purposes. Firstly, to gain information about the immediate effects of centrifugation, and secondly as a necessary background to the explantation experiments described later. The evidence from sections was amplified by simple dissection of live or fixed embryos under strong reflected light. This work was done before the normal table was published, but Nieuwkoop's terminology has been used throughout.

During the blastula stage the roof of the blastocoele is several cells thick. The outer layer, the superficial animal layer or coat layer, is one cell thick, the inner animal or sensorial ectoderm layer is two to three cells thick at the animal pole and thicker towards the equator. This layer forms the inner surface of the blastocoele roof about the animal pole, the cells are a grey colour by reflected light and possess some pigment. Moving downwards, the inner animal layer is covered on its inner surface by a layer of larger white cells completely devoid of pigment and forming a thick band above the equator. These are the marginal or presumptive mesoderm cells. They merge with the endodermal cells of the blastocoele floor in the equatorial region, the thickness of which is emphasised by the blastocoele floor being saucer-shaped with its deepest point in the middle.

About half an hour before the dorsal lip appears the pre-gastrulation movements get under way and bring about certain changes which are finished by the time a complete blastoporal ring is formed. The most striking result of these movements is that the blastocoele roof is completely cleared of marginal cells. These migrate downwards towards the equator and come to lie beneath the level of the blastocoele floor a little above the blastoporal lips. This process causes an expansion of the blastocoele and

makes the blastocoele floor quite flat. A groove forms round the periphery of the blastocoele floor, a little after the dorsal lip is visible, that separates the blastocoele floor from the inner animal ectoderm. This groove extends downwards to a little above the blastopore. The marginal cells then lie beneath and on the inside of this groove. This is shown by the difference in behaviour of ectodermal explants taken from late blastulae and very early gastrulae and those from gastrulae a little before the blastoporal ring stage. Ten explants were made from the former stages where the blastocoele floor was fairly flat and the downward movement of mesoderm was incomplete. The cuts were made at the level of the blastocoele floor and included of course marginal cells. All explants developed mesodermal structures and large inductions. In contrast with these, control explants can be taken from early gastrula stages up to a little after the complete blastoporal ring stage, provided the cuts are not made further vegetally than the bottom of the groove separating the inner animal layer from the blastocoele floor. Neither must the explant be contaminated with any material on the inside surface of this groove or mesodermal structures and inductions will result.

Bearing in mind that the pre-gastrulation movements are not finished at the time the dorsal lip is formed, the situation in the early gastrula is as follows:

Over most of its area the blastocoele roof is two cells thick, but above and to the sides of the dorsal lip the sensorial ectoderm is four to five cells thick, and the whole of the neural plate will be formed from this region.

On the periphery of the blastocoele floor above the dorsal lip is a region of large cells clearly delineated in section; these are smaller than the endoderm cells and they contain more cytoplasm staining with toluidine blue, their nuclei are denser and stain more strongly also. These are the cells of the prechordal plate (Pasteels 1949). They form the inner lining above the dorsal lip of the previously mentioned grooves. The rest of the mesoderm lies at the bottom of the groove, and spreading a little on its inner face. This can be shown in sections by the staining properties of the marginal cells and in whole embryos by the colour and cell size of the different layers. The mesoderm is continuous around the embryo from the dorsal to the ventral lip, and the cell

size decreases caudally; this is correlated with the gradient in cell size seen along the axis of the neurula, the cells of the anterior trunk somites for example being much larger than those of the posterior somites. The mesoderm cells can be followed in sections during gastrulation because they are the smallest cells of the inner cell mass and move without reference to the adjacent sensorial ectoderm with which they might be confused in the ventral half. Lying on the surface of the early gastrula and forming a crescent of cells which is the first to be invaginated is a region of endoderm very clear in sections (Pasteels 1950).

The key to the understanding of the gastrulation movements in *Xenopus* is to consider the position of the presumptive neural plate on the early gastrula. It is a crescent of cells lying above the dorsal and lateral blastopore lips and subtending an angle of no more than thirty degrees from the centre of the egg, in the plane of the animal vegetal axis. In the course of its formation it will expand posteriorly by enclosure of endoderm over an angle of ninety degrees, and anteriorly over an angle of about sixty degrees. This is a different state of affairs from the newt gastrula, where the anterior extremity of the presumptive neural plate is on the animal pole and the plate is formed solely by a posterior extension. The mesoderm of the *Xenopus* gastrula lies internally as a ring of tissue situated somewhat posteriorly to the middle of the future neural plate, subtending an even smaller angle with the centre of the egg. The extensions it must undergo are similar to that of the neural plate itself; it must extend further posteriorly than anteriorly. This again is in strong contrast with the newt gastrula where the neural plate extends posteriorly and the invaginated mesoderm only anteriorly, the two layers passing each other in opposite directions. Hence the *Xenopus* gastrula does not show the classical picture of mesodermal invagination movements and uni-directional migrations of cells for each presumptive tissue. The *Xenopus* embryo is already a three-layered embryo before the pre-gastrulation movements occur, and gastrulation is a matter of enclosing the endoderm and patterning an axis, by a fore and a hind extension of crescentic anlagen. How exactly regional inductors get sorted out to their right places in a process like this is a matter of speculation. Equally speculative at the moment however

must be any conclusion that *Xenopus* possesses regionally specific inductors. This will be dealt with in the discussion. No mention has been made of convergence movements towards the mid-line though they must certainly occur, both in the formation of the neural plate and the mesoderm.

Again, in contrast with the newt, it should be borne in mind that archenteron roof is not identical with the mesoderm underlying the neural plate. Very little invagination of the archenteron takes place until the small yolk plug stage, when the process is suddenly taken in hand and completed in a very short period of time.

There are differences between the surface coat of *Xenopus* and the newt. The surface coat of the newt is a continuous layer on the surface of the embryo contributed to by all superficial cells irrespective of presumptive fate. The coat of *Xenopus* can be considered as a specialized layer of ectodermic cells with limited potentialities. Coat cells do not form sensory tissue, they form only the ependyma of the neural tube, a ciliated epithelium similar to the epidermis on the surface of the embryo. This layer of cells can be stripped off the early gastrula, free of sensory cells, and is incapable of neural differentiation when combined with organizer. This layer appears to be unique among the amphibia (see Nieuwkoop).

Prior to the pre-gastrulation movements the mesoderm lies in a thick and wide crescent above the future position of the blastopore lips. As regards the two-dimensional distribution of presumptive mesodermal areas, this stage may be exactly comparable to the newt late blastula or early gastrula. This cannot be determined by making an orthodox fate map of *Xenopus* because that is impossible, the marginal cells in their internal location are not available for vital staining.

The most promising method would seem to be a localized labelling with tracer. Sirlin (1956) used the well-known fact that a crescent of endoderm lies on the surface of the embryo just above the position of the dorsal and lateral blastoporal lips, to try out the utility of this technique, which worked successfully. The author, in collaboration with S. Brahma (unpublished), carried out a small-scale experiment to see how successfully this technique could be extended to studying internal layers. Small groups of some half dozen labelled mesoderm cells were implanted into the presumptive mesoderm of early gastrulae. Two grafts, slightly to one side of the dorsal lip, were recovered in the anterior trunk

somites on the correct side of the neurula; eight other grafts were made on embryos about the ventral lip, with the idea that this would test the technique more precisely than the former grafts as the mesoderm is thinner ventrally; all these embryos gave large secondary inductions indistinguishable from those produced by centrifugation. This surprising result has been confirmed with unlabelled cells and with suitable controls, (also Schechtman above). In the light of the last experiment the following improved method is proposed: labelling by inserting a fine glass tube containing a small plug of labelled agar at one end. This could be stuck in the embryo like a microelectrode in any desired position. When making the autoradiographs, cut thick sections and over-develop the film.

Before concluding this section something must be said about terminology. Two distinct sets of terminology are in use for the different layers and tissues of the *Xenopus* embryo. One series giving such terms as superficial animal layer, inner animal layer, etc. has been mentioned here but not used, as they really concern the recognition of particular plasms in early development. The other series of terms, such as superficial ectoderm and sensorial ectoderm, connects the former terminology with presumptive fate. Marginal tissue is primarily a term of the former group which Nieuwkoop further subdivides, but which has been used here as a convenient term of the first order for presumptive mesoderm. This dual terminology need lead to no confusion, and is necessary until the exact fate of all layers is precisely known.

The terms such as late blastula and gastrula are more difficult. If gastrulation is defined as the process by which a two-layered embryo becomes three-layered, gastrulation cannot be said to occur at all in *Xenopus*, because it appears to be three-layered from a very early stage. The newt gastrula has always been the textbook example of amphibian development, and such useful and time-honoured terms as blastula and gastrula are unlikely to fall out of favour when discussing *Xenopus* development just because they do not mean quite the same in the two cases. But there is a real danger in a case like this of misunderstanding and ambiguity. The terms blastula and gastrula, with their subdivision of early, medium, and late, can be retained as superficial descriptive terms

in *Xenopus* development; if they are so limited they correspond exactly to their same use in newt development. Limited thus, the various stages of gastrulation defined by the progress of the blastopore refer solely to the degree of endodermal enclosure. Where the terms are used to describe the extent of mesodermal invagination or archenteron formation, they are no longer comparable.

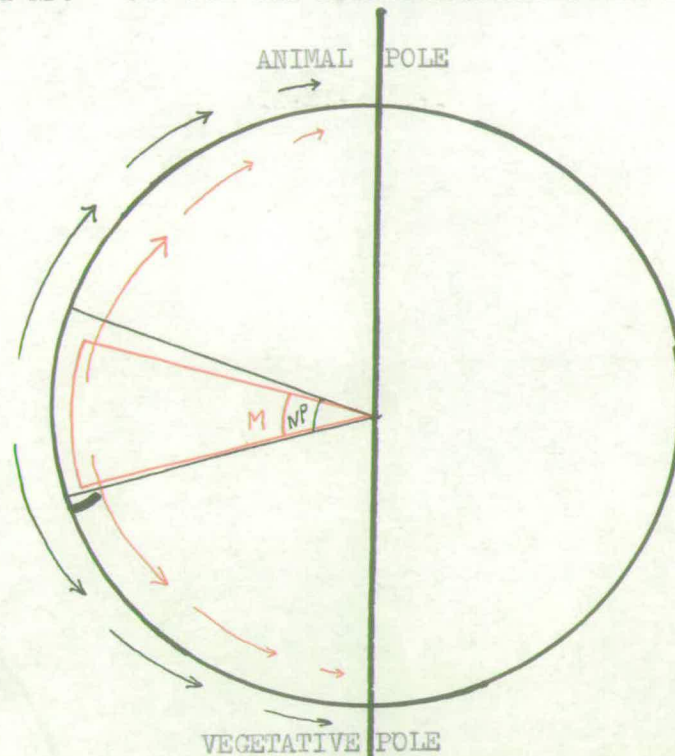
The term 'pre-gastrulation movements' is reasonable so long as the connotation of gastrulation is as above. Nieuwkoop (personal communication), using gastrulation in a wider sense as the formation of the axial pattern, considers gastrulation to begin before the dorsal lip is formed. In this case the pre-gastrulation movements mark the beginning of gastrulation and the term will have to be changed. It is the author's opinion that confusion is more likely to be avoided by limiting the term 'gastrulation' to describe the progress of the blastopore. In this case 'pre-gastrulation movements' should still be changed because they are by no means complete when the dorsal lip appears. But the field will be clear for the growth of a suitable and accurate terminology to describe *Xenopus* development without the unconscious restrictions imposed by trying to describe *Xenopus* in terms of the newt.

DIAGRAM COMPARING THE MORPHOGENETIC MOVEMENTS OF THE PRESUMPTIVE MESODERM AND THE PRESUMPTIVE NEURAL PLATE DURING GASTRULATION IN AN URODELE AND IN XENOPUS LAEVIS.

Schematized vertical sections through the dorsal lip of the early gastrulae. The segments subtended by the mesoderm in relation to the centre of the embryos are marked in red, the angle with the centre is marked M. The neural plate is marked in black and the angle labelled NP. The red and black arrows denote the directions of the movements of the respective layers during gastrulation.

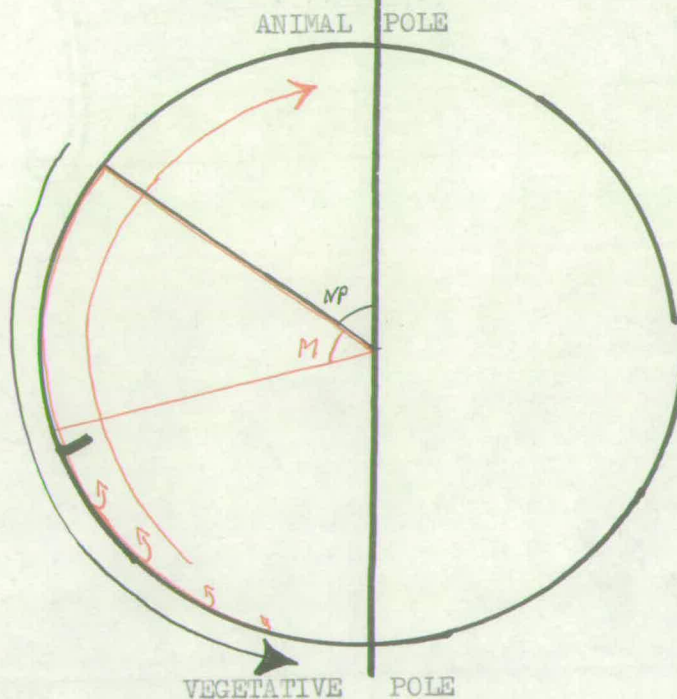
XENOPUS

Dorsal lip



URODELE

Dorsal lip



THE IMMEDIATE EFFECTS OF CENTRIFUGATION

It is convenient to describe the effects of centrifugation on the early gastrula, although it is not the most sensitive stage, because the various presumptive elements are fairly distinguishable even after treatment.

The gross changes of form are understandable on the basis of a movement of lighter elements centripetally and heavier ones centrifugally. Embryos orientate themselves in a centrifugal field with the lighter animal pole centripetally. The blastocoele fluid, being lighter than embryonic tissue, is driven out of the embryo and collects in the perivitelline space above the animal pole. The blastocoele roof moves correspondingly in the opposite direction and is pushed down on to the blastocoele floor. This tissue was originally a hollow hemisphere, and is now a flat plate of the same diameter, thrown into folds and crinkles to accommodate itself to a reduced area. The inner sensorial ectoderm of the blastocoele roof is split in places, and often wide fluid-filled spaces intervene between the outer coat ectoderm. These injuries are caused by the passage of blastocoele fluid to the exterior, the sensorial ectoderm possessing poor healing properties. Small holes in the outer ectoderm are very quickly repaired and leave no trace due to the amazing healing properties of the surface coat. Embryos centrifuged very strongly show white patches on the animal pole which consist of a small plug of inner cells protruding through a hole in the coat.

The presumptive mesoderm of the early gastrula can be recognized by cell size and relative yolk content provided it is in a normal state and the various tissues are in their correct positions. Inside centrifuged embryos considerable distortions have occurred and the true state of affairs can only be sorted out with the help of another criterion, in this instance the uptake of toluidine blue at a pH of 3.5. At this pH toluidine blue is reported to colour nucleic acids only. Mesoderm takes up less of the dye into the cytoplasm than the ectoderm which stains rather intensely; endoderm takes up stain only in the nucleus which is large and contains much nuclear sap.

Sections of freshly centrifuged embryos show that the presumptive mesoderm lying around the equator becomes shifted to lie on the peripheral region of the blastocoele

floor. The heavy endoderm moves centrifugally with respect to the lighter ectoderm. Mesoderm is intermediate in density between these two layers (judged on yolk content) and tends to assume a position between the two. By the early gastrula the groove round the circumference of the blastocoele floor reaches downwards to a little above the blastopore. The mesoderm lies at the bottom and on the inner surface of this groove. During centrifugation this groove becomes partially or wholly obliterated depending on the degree of treatment. The most reasonable explanation is to assume a strong tendency for endoderm to pile up at the vegetal end of the embryo, during which process the endoderm on the blastocoele floor, and the mesoderm which lies peripherally but at a more vegetal level, change their relative positions. The blastocoele floor sinks downwards, the periphery rises, obliterating the groove and bringing mesoderm on to the outer surface of the blastocoele floor directly underneath the depressed ectoderm of the blastocoele roof. This is exactly the state of affairs also in a centrifuged blastula before the pre-gastrulation movements have really got under way. In fact the mesoderm of the early gastrula undergoes a reversal of the pre-gastrulation movements. This will be discussed again later.

Yamada (personal communication) has observed somewhat similar shifts in centrifuged embryos of *Bufo vulgaris*.

The immediate effect of centrifugation therefore, is to obliterate the blastocoele, bringing the blastocoele roof, folded and wrinkled, in contact with the floor. Internal shifts have brought mesoderm on to the periphery of the blastocoele floor.

FURTHER DEVELOPMENT OF CENTRIFUGED EMBRYOS

Over most of the blastocoele roof the coat ectoderm and the sensorial ectoderm are each one cell thick. The sensorial ectoderm is thicker about the equator above the blastopore lips. This thickened crescent is widest above the dorsal lip where it subtends an angle of maximally thirty degrees in the animal vegetal axis, measured from the centre of the egg. This angle is very much reduced over the ventral half of the embryo. It is obvious that the area of ectoderm only two cells thick cannot partake in the epibolic movements which will enclose the endoderm and form the neural plate; these results accrue

from the extension of the thickened crescent above the equator. This thickened crescent of ectoderm is not visibly distorted by centrifugation, partly owing to its vertical position in the centrifugal field, and partly to the rigidity conferred by thickness. Thus in newly centrifuged embryos the flattened blastocoele roof appears to be bounded by a slightly raised rim. The pattern of wrinkles and folds already referred to is confined to the ectoderm only two cells thick, and the pattern maintains its identity until the neurula stage, as it is not affected by epibolic movements or stretching. Thus the ectoderm of the early gastrula, which is pressed into contact with interior layers by centrifugation, can be followed by a clearly visible criterion to the neurula stage. The ectoderm, playing no role in gastrulation, keeps the same relative position throughout the process. That is to say, it remains anterior to the cephalic margin of the neural plate. As the neural plate stretches to subtend an angle of one hundred and eighty degrees over the surface of the neurula, the blastocoele roof ectoderm will form part of the ventral surface of the neurula. In normal development this movement can be followed by observing the position of the blastocoele up to the time of its obliteration.

Secondary inductions arise from the ectoderm of the depressed blastocoele roof.

Pasteels emphasises that secondary inductions only arise after the blastocoele roof has been permanently depressed. Sometimes the folds and wrinkles of the centrifuged ectoderm disappear during gastrulation, the blastocoele becomes reconstituted and the embryo assumes a normal appearance. Inductions do not arise in such embryos.

THE SIZE, POSITION AND STRUCTURE OF SECONDARY INDUCTIONS OF THE FIRST SPECIES.

One species of inductions contains a component originating from the posterior ventral region of the neurula and consisting of a tail-like appendage containing spinal cord, notochord, and some mesenchyme which may or may not be condensed into somite blocks. In many cases this is the total extent of the secondary formation, as such inductions are correlated with the lowest level of significant treatment. Higher levels of treatment bring progressively more anterior regions of the ventral surface into the secondary field.

A frequently occurring type of induction is similar to that just mentioned, with the addition of an anterior nervous component which leaves the ventral surface and joins the primary hindbrain. This interaction of primary and secondary axes involves certain changes in the mesoderm, and the pronephros is often greatly enlarged on the side in question. Inductions of this type seem to have been the largest which arose in Pasteels' material, and are the largest inductions produced by centrifugation to be described in any detail. It is this which has led to the generalization that centrifugation produces only rhombencephalic and spinocaudal inductions. Larger inductions may have cropped up in Karasaki's and Yamada's material.

But the involvement of more anterior regions of the ventral surface of the embryo in the secondary field does not cease at a level opposite the primary hindbrain. After a high level of treatment the whole of the ventral surface of the head may be included, up to the anterior extremity. In such cases the anterior extremity of the head is filled by a large prosencephalic cavity which immediately bifurcates posteriorly into a dorsal primary axis and a ventral secondary axis. The primary axis is always to a greater or lesser extent microcephalic, the secondary axis extends along the ventral surface of the embryo and usually ends in a secondary tail growing out from the posterior region. The secondary nerve cord is almost always sufficiently well organised for the fore-, mid-, and hindbrain to be identified. The symmetry follows that of the primary axis, such that the secondary nerve cord is a mirror image, albeit (usually) imperfect, of the primary axis. This mirror image symmetry is a constant feature of head inductions.

THE EXPLANATION OF MIRROR IMAGE SYMMETRY IN HEAD INDUCTIONS

1. The whole of the secondary axis, from the anterior extremity of the head to the secondary tail posteriorly, forms from ectoderm that was originally the blastocoele roof of the early gastrula.
2. The ectoderm above the ventral blastopore lip forms the posterior ventral surface of the neurula, ectoderm nearer the dorsal lip finishes up in a progressively more anterior position on the ventral surface of the neurula.

3. Ectoderm is not determined at the late blastula or early gastrula stage, therefore the secondary inductions must be caused through contact with mesoderm.
4. Therefore the mesoderm above the ventral blastopore lip is a causal agent in the development of secondary tails on the posterior ventral surface of the neurula. Mesoderm about the dorsal lip is likewise concerned with anterior inductions in the head.

There are two ways in which mesoderm may play a causative role in the formation of secondary inductions. It is necessary to distinguish these clearly:-

- a) The mesoderm acts as an inductor upon the blastocoele roof ectoderm when the two make contact on centrifugation. The mesodermal component of the induction is also an induction in ectoderm or arises by the incorporation of adjacent host tissue into the secondary individuation field - whatever that may precisely mean.
- b) Some of the mesoderm gets trapped under the blastocoele roof and takes no part in the morphogenetic movements of gastrulation. It self-differentiates on the ventral surface of the neurula and induces nervous tissue in the ectoderm.

Evidence for a) is very strong, including the induction of mesoderm. This is dealt with fully in a later section.

However there is also good evidence for b). Embryos with all but the smallest posterior inductions show microcephaly of the primary axis culminating in a complete absence of sense organs in some embryos bearing head inductions. Microcephaly is partly due to a generalised response of the embryo to injury, but in some of these embryos sense organs absent from the primary axis are found associated with the secondary axis in the ventral region of the head. Thus the primary axis may lack all trace of optic vesicles and these may be found immediately above the adhesive gland, on the ventral surface of the head, associated with the secondary midbrain (Embryos BC16-1, BC15-3, see illustrations). Thus the secondary brain may be larger and more complete than the primary brain. This clearly favours alternative b) above. In these cases a part of the presumptive head organiser above the dorsal lip may become trapped under the blastocoele roof (bearing in mind the mesodermal shifts described earlier) and finally occupy a position more anterior

to the anterior margin of the neural plate, that is above the ventral surface of the head. This would explain the strong primary microcephaly and the large secondary brain. This explanation is favoured by another fact. Eyes associated with primary axis are almost always superficial even when the axis is strongly microcephalic. In those embryos where the eyes are associated with the secondary axis, they usually lie internally, and this irregularity is not surprising if they are to be induced from mesoderm brought somewhere above the ventral surface of the head.

A more direct proof that tissue can become trapped beneath the blastocoele roof comes from explantation work. It is often quite impossible to make clean ectodermal explants from strongly centrifuged, early gastrulae which later develop the largest head inductions. The underlying tissue is so closely adherent to the ectoderm that these explants cannot be freed from mesodermal contamination. Furthermore it is sometimes found that the folds and wrinkles into which the ectoderm is thrown on centrifugation grasp portions of the underlying tissues so as almost to enclose them. This is a strange fact and difficult to rationalise.

INDUCTIONS OF A DIFFERENT SPECIES SUPERNUMERARY AXES BY A FRAGMENTATION OF THE MESODERMAL FIELD

All the inductions so far described are of the same species. They all arise from the blastocoele roof and show some sort of mirror image relationship to the primary axis. There is another series of inductions which only occurs in a low frequency after strong treatment. These owe their formation to a radical disturbance of the normal gastrulation movements. Two, three or even four axes may be formed, they are quite separate anteriorly in extreme cases but are always fused posteriorly into a single axis. This is exactly opposite to the inductions of the type first described, where the primary and secondary axes were confluent anteriorly and ended posteriorly in quite separate tails.

Examples of this second type of induction are illustrated in embryos GC50-1 and GC50-6.

Two extreme embryos will be described:

GC11-1. The endodermal mass is roughly spherical and most of it is unenclosed by

mesoderm and ectoderm. Raised off the endoderm at one point is a blind finger-like protuberance containing a large nerve cord, eye tissue, a notochord, the whole enclosed in mesenchyme. This axis is continued posteriorly and gradually sinks into the yolk. Another small axis fuses with the first posteriorly, to give a wide plate of nervous tissue, the lateral extremities of which are underlain by the two axial notochords which remain separated. No auditory vesicles, heart, pharynx, pronephros, and adhesive gland.

GC11-2. Similar to GC11-1 but has also two small additional axes running along what may be the ventral surface of the embryo. All four axes fuse into a single nerve cord and notochord posteriorly.

The embryos just described are extreme cases of this type of induction. However there is a whole spectrum of lesser abnormalities connecting these embryos with the normal.

Further down the list come embryos in which two distinct axes run side by side along the dorsal surface of the neurula. Two-headed animals are never found; the axes are fused anterior to the rhombencephalon as well as posteriorly. This really amounts to a single axis duplicated in certain parts. Sometimes the duplication is only partial in the transverse plane. For instance a trunk section may show two spinal cords and notochords and only three ranks of somites, the centre one contributing to both axes. Moving closer to the normal condition one finds embryos in which the notochord is duplicated or the nerve cord is enlarged and divided by a vertical septum, over a distance of only one or two sections. All embryos bearing this type of induction are strongly microcephalic, sense organs are often absent.

This latter type of induction is quite distinct from the type which forms from the blastocoele roof and there are no cases which might be considered intermediate between the two types. This strongly suggests that different causes are responsible for the two species.

The most extreme examples of species 2, the embryos GC11-1 and 2 and GC50-1 must have been caused by an abnormal distribution of mesoderm during gastrulation, out of which arose two or more individuation fields. In these cases the individuation fields are axial

fields, but in the more normal embryos organ fields. In this manner can all inductions of species 2 be explained.

DEFECT ANOMALIES AND MICROCEPHALY

Embryos bearing small posterior inductions from the blastocoele roof are otherwise perfectly normal. Those bearing progressively larger and more anterior inductions show an increasing microcephaly, and deficiencies in the primary axis are never wholly made up by the secondary axis. All embryos bearing the second type of induction are strongly microcephalic as are those few embryos from a strongly centrifuged batch of eggs which do not produce inductions. The degree of microcephaly is correlated with the degree of centrifugation, and, as far as they can be compared, is not different in degree between embryos with and without inductions. Hence although microcephaly and the magnitude of the secondary structures are correlated, because both depend on the level of centrifugation, they appear to be independent in their development. Microcephaly appears to be the generalized response of the animal to injurious treatment by centrifugation. Secondary inductions are the result of the particular distortions inside the embryo, and become superimposed on the generalized response.

This idea is corroborated by the fact that microcephaly of an identical kind is a common reaction of *Xenopus* embryos to all sorts of injurious conditions; for instance, U.V. irradiation, overcrowding, extreme temperatures, mechanical injury, and indeed under all injurious circumstances.

The microcephalic syndrome for a tadpole two days after hatching may be summarised as follows:

A. External Characters.

- i) Eyes small to absent, poorly pigmented.
- ii) Head small, narrow in transverse section.
- iii) Adhesive gland always present, often protuberant.
- iv) Head and trunk poorly extended in the anterior posterior line.

B. Internal Characters.

- i) From iv above. Head structures often underlain by nutritive endoderm.

- ii) Chordalization of the prechordal plate, chorda underlying forebrain.
- iii) Auditory vesicles and heart often reduced or absent.
- iv) Head sections have a solid appearance - organs are packed close together, cavities tend to be small or reduced.

RECAPITULATION

The various abnormalities in development produced by centrifugation of the late blastula and early gastrula stages arise through the interaction of three different causes.

1. An unspecific response of the animal to injury. Not apparent in lightly centrifuged embryos, but found in a pure form in the few embryos not producing inductions after strong centrifugation. This response involves the microcephalic syndrome.
2. Induction of a secondary axis from the blastocoele roof. The most frequently found abnormality, exhibited weakly but in a pure form in embryos bearing secondary tails.
3. Fragmentation of the axial individuation field leading to partial or complete duplication of axis or parts of organs. Only arises in strongly treated embryos.

1 and 2 occur by themselves, 2 and 3 are mutually exclusive, 3 only becomes apparent at a level at which 1 is operating.

2 and 3 appear to be mutually exclusive alternatives, because no embryos have been found bearing both, and this is good evidence that they originate in different ways. The distortions in the embryo which bring about these two changes must be quite different, and which species of induction results may be due to the exact orientation of the embryos in the centrifugal field. This is not meant in contradiction to the general statement on orientation above, but the exact shape of the embryos can vary to a certain extent and this may be important in determining the precise species of distortion that occurs on centrifugation.

THE EXPERIMENTAL APPROACH1. ATTEMPTS TO PRODUCE INDUCTIONS BY OTHER MEANS BESIDES CENTRIFUGATION.

As stated earlier, grafts of some half a dozen mesoderm cells taken from above the dorsal lip and grafted into the mesoderm above the ventral lip, cause the formation of well-formed secondary tails from the posterior ventral surface of the neurula. These inductions are very similar to those produced by centrifugation. Controls that had undergone the same operational procedure without the implantation of any tissue, developed normally.

Attempts to place the blastocoele roof in firm contact with the floor by other means besides centrifugation have failed, although Schechtman claimed success with *Hyla regilla*. Pressure by means of glass slides or steel balls distorted the embryos but did not cause the blastocoele fluid to collect in the perivitelline space. Sometimes some of the blastocoele fluid escaped through a hole in the vitelline membrane. These embryos never grew inductions, but some bore thickened ectodermal patches on the ventral surface and in some others the mesoderm was unequally distributed between the two sides of the embryo.

In another attempt, the embryos were chemically decapsulated, and the vitelline membrane removed. The blastocoele roof was opened with forceps and the fluid expelled by pressing the roof on to the floor. These embryos developed normally, the reason being that the depression of the blastocoele roof was not permanent as in time the blastocoele became at least partially reconstituted. In most of these embryos there was some mesodermal assymetry, and large thickened pads of ectoderm on the ventral surface of the neurula. This latter remained undifferentiated, except in one embryo which bore an induction, and corresponded to Dalcq's "ectoderm neutralisee".

All these experiments failed through lack of a firm permanent contact between the blastocoele roof and the floor. Even if a permanent contact was made in some embryos, secondary inductions might be lacking because there is no substitute for centrifugation in reversing the pregastrulation movements.

This suggests that it is the pressure of the blastocoele fluid in the perivitelline space above the animal pole that maintains the contact in centrifuged embryos. If so,

the removal of the vitelline membrane from a batch of newly centrifuged embryos should lower the frequency of inductions. This happens in the case of embryos which have not been treated very strongly. But the experiment is defective because after centrifugation it is all but impossible to remove the vitelline membrane without making a hole in the embryo. The blastocoele fluid collected in the perivitelline space above the animal pole protects the embryos from injury by forceps, but when the pressure inside the vitelline membrane is released, the tissue on the animal pole usually tears, probably due to a sudden albeit slight expansion of the embryo. The blastocoele roof, it is worth remembering, has already been damaged by the exodus of the blastocoele fluid. Thus the inside of the embryo is exposed to the external medium. We infer from this experiment that any procedure that assists the reconstitution of the blastocoele in centrifuged embryos lowers the frequency of inductions, but we cannot infer that the pressure of the fluid in the perivitelline space is necessary in maintaining the contact between blastocoele roof and floor, although such an assumption seems most plausible.

2. THE EFFECT OF DECAPSULATION, AND OF CHANGES IN THE EXTERNAL MEDIUM, UPON THE PROPORTION OF SECONDARY INDUCTIONS PRODUCED BY A GIVEN DOSE OF CENTRIFUGATION.

If the proportion of secondary inductions produced by a given dose of centrifugation on embryos from the same ovulation, depends only on the degree of intra-cellular stratification, the proportion should remain the same in undecapsulated and decapsulated embryos. If, on the other hand, the proportion of inductions depends on the efficiency with which the blastocoele roof is thrown into permanent contact with mesoderm on the floor, then decapsulation, and pre- and post-centrifugation treatments with strong saline solutions may change the frequency of inductions by altering the rigidity of the embryo, and by an osmotic mechanism.

In this experiment, see Table I, series A, a batch of embryos was decapsulated one hour before centrifugation. One lot was placed immediately into two and a half strength Holtfreter solution, the rest remaining in one tenth Holtfreter. They were centrifuged with a control batch of undecapsulated embryos from the same lot. After centrifugation the embryos in two and a half strength Holtfreter were transferred to one tenth Holtfreter

and the other decapsulated embryos placed in two and a half strength Holtfreter for one hour.

The undecapsulated embryos gave nearly one hundred percent of inductions.

Pre-treatment with two and a half strength Holtfreter solution might be expected to reduce the proportion of inductions by causing water to move into the embryos when they were returned to one tenth Holtfreter after centrifugation. The proportion of inductions is reduced to fifty percent in these embryos.

TABLE I

The effects of decapsulation and treatment with hypertonic Holtfreter solutions on the frequency of secondary inductions within a single ovulation. Embryos scored two days after hatching.

SERIES A

STAGE	CENTRIFUGATION		UNDECAPSULATED			DECAPSULATED		
	g.	mins.	No.	Sec. I.	% Sec. I.	No.	Pre-treatment Sec. I.	Sec. I.
LB	670	4	37	36	97	48	27	56

Pre-treatment: to $2\frac{1}{2}$ strength Holtfreter solution for one hour before centrifugation, thence to .1 Holtfreter solution.

Frequency of inductions in undecapsulated embryos compared with pre-treated embryos
 $\chi^2 = 17.72, \quad p. < .001$

TABLE II

The effect of increasing the period of centrifugation on the frequency and grade of inductions correlated with microcephaly, and where the level of g. is not limiting.

Stage	Centrifugation		Total	% Sec. I.	Column 1		Column 2		Column 3		Column 4	
	g.	mins.			Normal		Two rudiments		One rudiment		Eyes absent	
LB	480	2½	18	28	5	13					5	
		3	28	93	16	1	9	1	1			
		4	53	98	8		42	1			2	
		6	31	87	3	2	16		2		6	2
		7	8	100	3		2		2		1	
		10	33	85	4		12	3	3	1	9	1
			171		39	16	81	5	8	1	23	3

In Columns 1, 2, 3, and 4 the figures on the left refer to embryos with inductions; those on the right refer to embryos without inductions.

See Table 2. Embryos centrifuged at the late blastula stage at 480g for times varying from two and a half to ten minutes. Tadpoles two days post-hatching ^{were} initially scored for the microcephalic syndrome and divided into four groups. Within each group they were further scored for secondary inductions. The degree of microcephaly was assessed on the presence or absence of eyes.

The use of a simple, objective criterion like this rules out a subjective assessment which would differ from one worker to another and is apt to vary within a single person's observations. The eyes are a very sensitive measure of the microcephalic syndrome and are the first organs in the embryo to be affected. If the eyes are normal the animal is not microcephalic. The first column headed "normal" contains all the embryos with normal eyes. The second column headed "two rudiments" contains embryos having two eyes

where one or both are reduced. This is an unavoidably mixed group that contains practically normal embryos at one extreme and strongly microcephalic embryos showing two visible eye rudiments at the other extreme. The third column headed "one rudiment" is composed of more strongly microcephalic embryos where only a single eye rudiment is visible externally. The fourth group calls for no comment. The figures on the left in each column refer to embryos bearing inductions, the figures on the right to the embryos without inductions.

The grade of induction is closely correlated with the degree of microcephaly. All inductions under Column 1 are small secondary tails. Column 2 includes principally head inductions of the same type, that is originating from the blastocoele roof. Columns 3 and 4 also include some embryos of this type and those produced by a fragmentation of the individuation field.

The figures reveal the following points:

1. The lowest application time of two and a half minutes produced few inductions.
2. The higher application times produced a maximal frequency of inductions - 85% to 100%.
3. The longer the application time, the higher the proportion of microcephalics and the greater their severity.
4. Animals without inductions are found at all levels of treatment, and may show any degree of microcephaly.

The results of these experiments show that the proportion of secondary inductions obtained from a given treatment on a batch of embryos can be significantly altered in ways which cannot effect the degree of intracellular stratification. Furthermore the results can be explained by assuming that any treatment which alters the rigidity of the embryos (decapsulation) or the water intake or output, can favour or prevent the partial reconstitution of the blastocoele, which in turn affects the contact between blastocoele roof and floor. The conclusions drawn from this experiment therefore are against Pasteels' theory of inductions by intracellular stratification, and favour an inductive mechanism.

3. THE DEVELOPMENT OF THE ECTODERM OF THE BLASTOCOELE ROOF EXPLANTED FROM EARLY GASTRULAE AFTER CENTRIFUGATION.

This experiment was started initially as a repeat of Pasteels' explantation experiment of the same type. However when it was found that explants taken from embryos immediately after centrifugation failed to develop neural and mesodermal structures, Pasteels' original procedure was extended to include the time variant. Thus the experiment includes the results from explants taken from embryos up to one hundred and eighty minutes after centrifugation.

In preliminary experiments on uncentrifuged embryos it was found that explants gave neurulisations when taken from late blastulae in which the pre-gastrulation movements were not well advanced. This means that late blastulae will only give reliable control explants if they are only a very short time away from the beginning of gastrulation. In order to work with these stages it would be necessary to time their development very accurately at a constant temperature, knowing to within a few minutes the time at which they were layed. As the toads were customarily ovulated overnight this was most inconvenient. Therefore all explants were taken from the early gastrula stages, between the time the dorsal lip appears and a complete blastoporal ring is formed. This gives a period of a little under an hour for each embryo during which explants could be made. This is an absolutely reliable period for making control explants, and is defined at both ends by perfectly unambiguous external criteria. In fact no control explants on uncentrifuged embryos are necessary in this experiment because the nature of the results makes them superfluous. As the explants taken from embryos immediately after centrifugation do not develop neural or mesodermal tissue, they form a much better control for the remaining explants than would explants from uncentrifuged embryos.

To make explants from embryos at a series of times after centrifugation, a mixed batch of blastulae was centrifuged and explants were taken from those embryos which began to gastrulate after the desired period.

Control explants are made by cutting the ectoderm along the bottom of the groove bounding the blastocoele floor. In this way the explants are the largest it is possible

to make. However in centrifuged embryos the groove is partially or wholly obliterated. It has already been mentioned that the thickened ectoderm above the equator is not distorted by centrifugation and forms a raised rim around the central depression; in making explants from centrifuged embryos the cut was made immediately beneath the outer edge of this rim. The explants were not noticeably much smaller than those from uncentrifuged embryos.

The explants from centrifuged embryos were always removed with some mesoderm cells adhering. These were removed with tungsten needles or by a fine pipette. Mesoderm and endoderm cells can be readily distinguished from the sensorial ectoderm when viewed under a strong reflected light. The ectoderm is a very smooth layer of small grey cells, that are somewhat translucent. In comparison the mesoderm and the endoderm cells are much larger and contrast well enough with the ectoderm on this count alone. Besides this they are bright yellow in colour due to their yolk content and the fact that they are devoid of pigment. Their yolk content also renders them very opaque and they glisten in the light and reflect it strongly. For these reasons it is easy to spot every mesoderm or endoderm cell adhering to the newly explanted ectoderm, and it is usually a fairly easy matter to remove them with needles or a fine pipette. The explants heal very rapidly into balls.

It is quite impossible to make clean explants from very strongly centrifuged embryos, because of the large number of adhering mesoderm cells and the tenacious way they stick to the ectoderm. Bear in mind that the ectoderm tends to roll up into a ball very quickly and does not allow one unlimited time to clean the explant. Furthermore when the mesoderm cells are stuck very tightly to the ectoderm it is difficult to remove them with the pipette without making a hole in the ectoderm. Under these circumstances the explant can finish up looking like a starling shot through with a twelve bore. Therefore the aim is to centrifuge the embryos at a level which will produce a high proportion of low grade, i.e. secondary, tail inductions. In fact it was decided in this experiment to try and aim for an induction frequency of about a half, so that when explanting from embryos centrifuged over an hour previously, where the ectoderm develops neural structures and mesoderm, one half of the explants would act as controls for the other

half. This would make a better control than the explants taken soon after centrifugation as the time variant would be included. It is impossible to ensure this frequency in any one batch of centrifuged embryos because the variability displayed by different batches of eggs is very great. By experience it is possible to surmise whether a batch will be sensitive or insensitive, but there is still much room for error. The problem of variability between batches of eggs will be dealt with later. In the latter half of this experiment the induction frequency averaged about 64%.

Explants were cultured at 18°C in full strength Holtfreter solution for a minimum time of three days and a maximum time of four days. Intact early gastrulae hatch in less than two days at 18°C and by three days the eyes are fully pigmented. Ectodermal explants combined with organiser have developed brains, eyes, lens, notochord, somites, and adhesive gland after three days, showing that this period is quite sufficient for an explant to demonstrate its potentialities. However, it was thought desirable to let the explants in this experiment grow to a maximum of four days, thus allowing as wide a safety margin as possible. It was found that explants which developed only epidermis very rarely survived for four days. They tended to disintegrate during the third day. Thus all the explants in this experiment which did not develop neural tissue can be assumed to have been fixed during the third day of their growth. A number of explants disintegrated before three days or were partially disintegrated at the time of fixation. These explants do not appear in Table 3. Explants containing neural tissue and mesoderm usually lived for four days and were then fixed. In other experiments ectodermal explants combined ~~as~~ with organiser have been kept in a healthy condition for as long as seven days, but this longer period of growth does not seem to mean more differentiated explants. They are no better developed than explants kept for four days.

Description of explants from centrifuged embryos sectioned after culturing for three or four days.

a) Explants developing no neural or mesodermal tissues.

The majority of these explants are solid in appearance with sometimes one or two

vacuoles. A few contain a single very large vacuole in the centre of the explant bounded by two layers of cells corresponding to the coated and sensorial ectoderm. The rest of the explant consists of one or two solid phlanges or fingers as thickening of the vacuolar wall. The vacuole is usually empty but in unhealthy explants that have been cultured too long and about to disintegrate, the vacuole often contains a number of loose cells with strongly pycnotic nuclei. Presumably these are unhealthy cells of the vacuolar wall that have fallen into the cavity.

The surface area of the solid explants tends to increase. This is achieved by the formation of many finger-like projections from the surface of the explant which give it a rough corrugated appearance. When these get long enough they fuse one with another to enclose ramifying lacunae giving the outer regions a sponge-like character. This demonstrates the inherent tendency of the early gastrula coat layer to stretch. It would prefer to cover a voluminous vacuole, for it will stay healthy longer when it does so. Where this happens the sensorial ectoderm lining the vacuole sometimes becomes a ciliated epithelium, and small pieces of tissue introduced into the vacuole are vigorously churned around. This is strange because the sensorial ectoderm does not give rise to any ciliated derivative in the normal embryo. The ciliated epidermis and the epyndyma are both formed from the coated layer of ectoderm. Hence sensorial ectoderm cultured in ectodermal explants can manifest a state of differentiation not encountered in the normal animal.

Explants developing no neural or mesodermal tissues stain very poorly on the third day with toluidine blue at pH 3.5. If the stain at this pH is specific for nucleic acids this suggests a continuous decrease in the nucleic acid content of the cytoplasm after explantation.

b) Explants developing neural and mesodermal structures.

In explants containing developing neural tissue and mesoderm the latter are strongly stained, and continue to show a strong affinity for toluidine blue until the explants become aged and the nuclei pycnotic. These explants are always vacuolated though the

size of the vacuole varies considerably. Neural and mesodermal masses are usually associated with the vacuole and are most often found inside it, though usually joined at some point to the wall. The degree of neural differentiation is low. Spinal cord has been identified in eighteen explants. It is always small. In some explants it forms a regular tube, in others it is ~~the~~ mostly a solid structure, tubular in parts. Four explants contain small prosencephalic structures with eye cup; both are poorly developed, the latter is no more than an orientation of cells around a typical chorionic pigment accumulation. In two explants this primitive eye cup is found associated with neural vesicle without any identifiable prosencephalon at all. Neural vesicles have been found in thirteen explants. Mostly these are quite large masses and in one or two explants voluminous. They are clearly distinguishable from the surrounding tissue, stain more strongly, and have the structure of a typical low grade induction.

Notochord has developed in five explants and is small in size, except in D41 where it is massive; it is well-differentiated in all explants. Somite has been found in twenty-seven explants. Unlike the notochord it is usually present in quantity. In the majority of cases it consists of large structureless lumps of muscle, as well differentiated as is usual in explants. In four cases it is split up into somite blocks and contributes to a small but recognisable axis. Adhesive gland has arisen in twenty explants. Where present it is large and well-differentiated. This tissue is always a very conspicuous feature of explants combined with organizer and is the only structure induced in explants by mouse kidney. It often occurs in uncentrifuged control explants, and it is surprising to find so few cases in this series.

A noticeable feature of these explants containing neural tissue and mesoderm is that the two tissues do not necessarily occur together in adjacent parts of the same explant. The significance of this will be dealt with in the discussion, as will also the whole question of contamination.

Table 3 is divided into three parts.

Part 1 consists of explants taken immediately after centrifugation, that is within ten minutes of the end of treatment. Thirty-two explants comprise this series; one explant contains neural tissue and one other somite. If the embryos from which these

explants were taken had been left intact they would have given 54% with inductions. This figure is worked out from the figures given in the table for intact embryos from the same batches of eggs, allowance being made for the number of explants taken from each batch. Assuming that only embryos bearing an induction would give an explant with an induction, one would expect seventeen explants in this series to produce neural inductions.

It is concluded that explants from newly centrifuged embryos behave no differently from those from uncentrifuged embryos.

Parts 2 and 3 of Table III consist of explants taken at various periods from thirteen minutes to over three hours after centrifugation. They are placed in chronological sequence. The division between parts 2 and 3 is arbitrary and is made for convenience. Part 2 contains explants made before ninety minutes after centrifugation, and Part 3 the explants made after ninety minutes. As a glance at the table will show, the explants in Part 2 behave the same as those in Part 1, while the explants in Part 3 produce neural and mesodermal structures. The division at ninety minutes is not intended to represent a qualitative difference between the two series of explants.

Throughout this experiment the aim has been to achieve a level of centrifugation giving about fifty percent inductions, scored by external criteria, in intact control embryos. The average for the experiment has been around the desired figure.

It might be thought that results could have been obtained more quickly and with greater efficiency if explants had been taken throughout from embryos giving one hundred percent of inductions, and this is perfectly feasible. However there is the question of control explants to be considered, and this particularly applies to Part 3 of the table. These embryos were centrifuged as mid- to late blastulae and as is the case throughout the experiment, the explants were taken when they reached the early gastrula stage. This situation is rather different from that in Part 1 of the table, where the embryos were centrifuged at the early gastrula stage, therefore these latter explants are not the best control for those of Part 3 of the table. Thus by centrifuging at a level after which fifty percent of the embryos develop normally, about half the explants made are controls, though of course one has no means of knowing in fact which are control explants and which are not.

There are forty-two explants in Part 3 of the table, of which twenty-one produced neural inductions. Assuming that embryos which produced inductions and only these embryos would have produced explants containing neural tissue, the expectation is that twenty-four explants in the table would show inductions.

The conclusion drawn from this close correspondence between explants and intact embryos is that all embryos bearing inductions, and only these embryos, yield explants containing neural tissue when the explants are taken not less than ninety minutes after centrifugation.

Somite developed in a total of twenty-seven explants of which only 18 appear in Part 3 of the table; the other nine occur in explants appearing in Part 2 of the table taken between forty-six and eighty-eight minutes after centrifugation, of which there are a total of twenty-three explants. Assuming that the neural and mesodermal structures developing in explants are due to an inductive stimulus, it is concluded that the stimulus causing somite development passes quicker than the neural stimulus.

There is a total of twenty explants bearing adhesive gland in Parts 2 and 3 of the table, of which all but two occur in Part 3. It is assumed that the inductive stimulus causing adhesive gland to develop (said to be archencephalic) takes about the same time to pass as the neural inductor.

There is a total of six explants containing notochord, equally divided between Parts 2 and 3 of the table. The figures do not show the one-to-one relationship between explants and intact embryos in Part 3 which is shown by the other tissues mentioned. Any statement upon the rate of notochordal induction is unwarranted.

The small number of explants containing notochord may be correlated with the type of secondary induction produced on the intact embryos. A level of treatment producing fifty percent of inductions invariably gives only small secondary tails, and these were in a large majority in the intact control embryos of this experiment. Some larger inductions occurred in certain batches, see line 2 of Table 2, which came from the same batch as explants D64-67. The secondary tails are a low grade induction and the preponderance of spinal cord and somites in the explants corresponds well with them.

Such inductions seldom produce much notochord, and the chorda is frequently absent. Adhesive gland does not occur in secondary tails, but as has been said, it is common in all types of explant.

With the exception of the first batch, these remarks do not apply to eggs used in Part 1 of the table. Although the frequency of inductions was not 100%, the majority were head inductions, and the results say much for the reliability of the explantation procedure. To avoid repetition, this latter question is dealt with in the discussion.

THE VARIATION IN RESPONSE SHOWN BY DIFFERENT BATCHES OF EGGS TO CENTRIFUGATION.

This variability is such a striking feature that something must be said about it. It is best dealt with here because the figures in Table 3 relating to the intact embryos illustrate it very well. It will be noted that the intact embryos corresponding to explants D43-45 gave ninety-four percent inductions. A part of this ovulation was put overnight at fifteen degrees Centigrade and used next day for explants D47-48. Note that the same level of treatment gave only four percent of inductions. This effect of low temperatures has been confirmed on other batches of eggs. Apart from this one exception, all the embryos in the table were laid and reared at eighteen degrees Centigrade throughout.

It has already been mentioned that there are some purely empirical criteria for deciding whether a batch of eggs is likely to be sensitive or not. In principle it is a matter of determining how rigid the embryos are. In some batches of embryos the ectoderm is particularly rigid and the mesoderm compact, and the embryos can be dissected cleanly without making a mess of them. Other embryos are "sloppy", and in extreme cases almost disintegrate when operated on. These latter are of course useless for making explants. Rigid eggs are sensitive to centrifugation as a rule. Sloppy eggs are often very insensitive, but once a certain threshold has been passed tend to produce large numbers of head inductions at once, without going through a stage giving secondary tails. This is understandable on the basis of a lack of rigidity in the ectoderm, as once the blastocoele

fluid starts to be forced into the perivitelline space, the whole blastocoele roof tends to collapse as a sheet, giving a large surface of contact with the underlying layers.

Certain toads tend to lay a majority of sloppy or rigid eggs; this may be connected with the age of the females and the length of time they have been kept in captivity.

Behaviour of ectodermal explants taken at the early gastrula stage from centrifuged embryos, the period between centrifugation and explantation being varied from zero time to over three hours; and the induction frequency of intact embryos from the same batches.

EXPLANTSINTACT EMBRYOS

	Mins. after centri- fugation	Nerve cord	Noto- chord	Som- ite	Adhesive gland	Stage	Centri- fugation g. mins.	Total	Sec. I.	%	Remarks
CE1-1	0-5	-	-	-	-	VEG	480 4	11	2	18	
2	"	-	-	-	-	"	" "	"	"	"	
3	"	-	-	-	-	"	" "	"	"	"	
4	"	-	-	-	-	"	" "	"	"	"	
5	"	-	-	-	-	"	" "	"	"	"	
6	"	-	-	-	-	"	" "	"	"	"	
CE2-1	10	-	-	-	-	"	" "	"	"	"	Contaminated
2	"	-	-	-	-	"	" "	"	"	"	
CE5-1	0-5	-	-	-	-	"	" "	"	"	"	
2	"	-	-	-	-	"	" "	"	"	"	
CE8-1	10	-	-	-	-	VEG	480 10	20	16	80	
2	"	-	-	-	-	"	" "	"	"	"	
CE9-1	0-5	-	-	-	-	VEG	1500 5	27	20	74	
2	"	-	-	X	-	"	" "	"	"	"	
3	"	-	-	-	-	"	" "	"	"	"	
CE10-1	"	-	-	-	-	VEG	2000 5	20	13	65	
2	"	-	-	-	-	"	" "	"	"	"	
3	"	-	-	-	-	"	" "	"	"	"	
CE11-1	"	-	-	-	-	"	" "	"	"	"	Contaminated
2	"	-	-	-	-	"	" "	"	"	"	
CE12-1	"	-	-	-	-	VEG	2000 5	34	22	64	
2	"	-	-	-	-	"	" "	"	"	"	
3	"	-	-	-	-	"	" "	"	"	"	
4	"	-	-	-	-	"	" "	"	"	"	
5	"	-	-	-	-	"	" "	"	"	"	
6	"	-	-	-	-	"	" "	"	"	"	
CE16-1	"	-	-	-	-	VEG	480 6	21	18	88	
2	"	X	-	-	X	"	" "	"	"	"	
3	"	-	-	-	-	"	" "	"	"	"	
4	"	-	-	-	-	"	" "	"	"	"	
5	"	-	-	-	-	"	" "	"	"	"	
6	"	-	-	-	-	"	" "	"	"	"	

The proportion of secondary inductions expected in the explanted embryos is these had been left intact: 54%

Table III - Part 2

40.

	Mins. after centri- fugation	Nerve cord	Noto- chord	Som- ite	Adhesive gland	Remarks	Stage	Centri- fugation g. mins	Total	Sec. I.	%
D20	13	-	-	-	-			<u>D11 to 19</u>			
D37	16	-	-	-	-		LB	670 5	17	16	94
D28	16	-	-	-	-			<u>D20 to 27</u>			
D11	17	-	-	-	-		EG	670 5	16	7	44
D21	18	-	-	-	-			<u>D28 to 36</u>			
D29	20	-	-	-	-		EG	480 7	8	4	50
D38	22	-	-	-	-			<u>D37 to 42</u>			
D13	25	-	-	-	-		LB & EG	480 5	21	16	76
D30	29	-	-	-	-			<u>D43 to 45</u>			
D43-1	21	-	-	-	-		LB & EG	480 10	16	15	94
2	27	-	-	-	-			<u>D47 to 48</u>			
3	33	-	-	-	-		LB & EG	480 10	24	1	4
D39	30	-	-	-	-			<u>D54 to 55</u>			
D14	32	-	-	-	-		LB & EG	670 8	25	15	60
D22	33	-	-	-	-			<u>D57 to 58</u>			
D31	35	-	-	-	-		LB	670 8	24	14	58
D40	35	-	-	-	-			<u>D60 to 62</u>			
D15	36	-	-	-	-		EG	670 8	23	4	17
D23	40	-	-	-	-			<u>D64 to 67</u>			
D16	41	-	-	-	-		LB	480 3	28	26	93
D32	41	-	-	-	-			<u>D68 to 70</u>			
D17	46	-	-	-	-		LB	480 2½	18	5	28
D24	46	-	-	X	-						
D33	46	-	-	-	-						
D25	50	-	-	-	-						
D41	51	-	X	-	X						
D34	51	-	-	-	-						
D18	52	-	-	X	-						
D26	57	-	-	X	-						
D35	57	-	-	-	-						
D19	56	-	-	X	-						
D36	61	-	-	-	-						
D27	62	-	-	-	-						
D42	62	-	-	-	-						
D44-1	71	-	-	-	-						
2	81	-	-	-	-						
3	88	-	-	-	-						

The proportion of secondary inductions expected in the explanted embryos if these had been left intact: 62%

Table III - Part 2 (cont'd.)

41.

	Mins. after centri- fugation	Nerve cord	Noto- chord	Som- ite	Adhesive gland	Remarks
D60-1	57-67	-	-	-	-	
2	57-67	X	X	X	-	
D61-1	73	X	X	X	-	
2	73	-	-	-	-	
3	73	X	-	-	X	
D64-1	77-83	-	-	X	-	
2	77-83	-	-	X	-	
D62-1	90-100	-	-	-	-	
2	90-100	X	-	-	X	

Table III - Part 3

	Mins. after centri- fugation	Brain	Spinal cord	Neural vesicles	Noto- chord	Som- ite	Adhesive gland	Remarks
D65-1	92-97	-	X	-	-	X	X	
2	92-97	-	-	-	-	X	-	
3	92-97	-	-	-	-	X	X	
D62-1	90-100	-	-	-	-	-	-	
2	90-100	X	-	X	-	-	X	
D66 & 67-1	95-115	-	-	-	-	X	-	
2	95-115	-	-	-	-	-	-	
3	95-115	-	X	-	-	X	X	
4	95-115	-	X	-	-	X	X	
5	95-115	-	X	-	-	X	X	Contaminated
6	95-115	-	-	-	-	-	-	
D57-1	95-106	-	X	-	-	X	-	
2	95-106	XEC	X	X	-	-	X	
3	95-106	-	-	-	-	-	-	
D68-1	110-115	-	X	-	-	X	-	
2	110-115	-	X	-	-	X	X	Contaminated
3	110-115	-	-	-	-	-	-	Contaminated
4	110-115	-	X	X	-	X	X	
D58-1	113-123	-	-	-	-	-	X	
2	113-123	-	-	X	-	-	X	
3	113-123	EC	-	X	-	-	X	

	Mins. after centri- fugation	Brain	Spinal cord	Neural vesicles	Noto- chord	Som- ite	Adhesive gland	Remarks
D54-1	123-135	-	-	-	-	-	X	Contaminated
2	123-135	-	-	-	-	-	-	
3	123-135	-	X	X	X	X	-	
D70-1	135-140	-	X	-	-	X	-	
2	135-140	-	-	-	-	X	-	
D55-1	141-151	-	-	-	-	-	X	
2	141-151	-	X	X	-	X	-	
3	141-151	-	-	-	-	-	-	
D56-1	155-170	-	-	X	-	-	-	
2	155-170	-	-	X	-	-	-	
3	155-170	-	-	X	-	-	X	
D59-1	156-166	-	-	-	-	-	-	
2	156-166	-	-	-	-	-	-	
3	156-166	-	-	-	-	-	-	
D45-1	160-180	-	-	-	-	-	-	
2	160-180	-	X	-	X	X	X	
3	160-180	-	X	-	X	X	X	
D47-1	170-190	-	-	-	-	-	-	
2	170-190	-	-	-	-	-	-	
3	170-190	-	-	-	-	-	-	
D48	190-210	-	-	X	-	X	-	

Table III - Part 3 (cont'd.)

Total no. of explants	Neural tissue	Notochord	Somite	Adhesive gland	Total
	21	3	18	17	42
%	50	7	43	40	

The proportion of secondary inductions expected in the explanted embryos if these had been left intact: 57%

DISCUSSION

The first part of this thesis shows how the defect and accessory anomalies produced by centrifugation are due to three separable causes summarised on pages 22 and 23. Furthermore, the results summarised in Tables I and II show that insofar as the proportion of secondary inductions produced by a certain treatment on a batch of eggs can be changed by altering the environment, there must be important causative factors besides intra-cellular stratification (assuming the latter to be a cause) in the genesis of secondary inductions. These results do not require further consideration here except to note that Schechtman's suggestion (see introduction pages 3 and 4) is identical with the explanation offered in the text to explain the formation of the first type of induction described, namely the type arising from the blastocoele roof.

Pasteels criticises Schechtman's theory on three counts:-

- i. The position of the secondary inductions on the neurula precludes their development from material originally forming the blastocoele roof. The opposite is true in *Xenopus*. Furthermore the great significance which Pasteels attaches to his explantation experiments is hardly justified if the explanted region does not form the inductions on intact embryos.
- ii. The period of sensitivity to centrifugation bears no relationship to the chronology of normal induction. This need not be a relevant consideration. The exact timing of normal induction is not a matter of ectodermal competence and the organising properties of the mesoderm - these two factors co-exist to allow induction over a considerable period - but depends directly on the course of gastrulation and the cohesion of cell layers.
- iii. The inductions resemble those obtained by using chemical inductors and denatured tissues. (1951) Pasteels is of the opinion that centrifugation produces inductions by a similar principle to sub-lethal cytolysis, and the action of certain dyes (Beatty, Zeilinski and de Jong, 1939). The experiment of Karasaki and Yamada which demonstrated that sub-lethal cytolytic inductions in Anuran species sensitive to centrifugation are always archencephalic, contrasts strongly with the behaviour of Pasteels' explants made

immediately after centrifugation.

The chief subject of this discussion will be the explant experiments and their significance. Firstly, the experiments will have to be discussed in relation to the question of mesodermal contamination which has been left until this point. Pasteels and the author deny that the results of their respective experiments were due to a contamination of their explants with mesoderm. How valid is this claim? Both authors insist that explants must be carefully freed from non-ectodermal tissue, the implication being that both believe that inducing tissue lies beneath and in contact with ectoderm in centrifuged embryos. The only direct way of ruling out contamination completely would be the interpolation of a barrier between the two layers before centrifugation. This is impossible. Explants taken from centrifuged embryos are initially contaminated with mesoderm and require careful cleaning. Therefore it is up to the experimenter to design his experiment in such a way that he can adduce from it a large enough body of indirect evidence confirming the reliability of his decontamination procedures, and rendering the possibility of results by contamination unlikely.

In considering the author's experiment, there are the following arguments against results by contamination:

- i. Mesoderm cells adhering to ectoderm can be particularly easily spotted in *Xenopus*.
- ii. The time variant - explants taken from embryos more than ninety minutes after centrifugation produce inductions, those taken before do not. This is a strong argument. To sustain the possibility of results by contamination one could only assume that the longer contact allowed in embryos explanted after ninety minutes increased the strength of the adhesion of mesoderm to ectoderm, rendering the explantation procedure unreliable. There was no evidence at the time to suggest this possibility.
- iii. A 1:1 ratio was found between the number of secondary inductions in a batch of centrifuged embryos and the proportion of inductions developing in explants from the same batches more than ninety minutes after centrifugation.
- iv. Endodermal or mesodermal contamination was found in seven explants. Four of these from Parts 1 and 2 of Table III developed no induction. The three from Part 3 were

quite typical of their type. This suggests that a degree of contamination too small to have any significant effect could be resolved by microscopic examination.

v. The neural inductions were of a low grade. If the mesodermal structures developing in the explants had been due to a self-differentiation of contaminating presumptive mesoderm one would have expected much larger and well-differentiated neural inductions. See Illustration of D41 for example. In fact the mesodermal component in these explants is manifestly ectodermal derivation, for the cells are very small and contain very few yolk platelets; true mesoderm contains many.

vi. The neural tissue and the mesodermal differentiations do not necessarily occur side by side in the explants as they would have to on the assumption that one induced the other. This argument is somewhat weakened by the fact that explants change their shape to some extent during their growth.

None of these arguments taken alone proves that contamination is not responsible for the results obtained. But taken together they constitute, in the author's opinion, a body of indirect evidence sufficient to render the contamination hypothesis unlikely. Therefore it is considered reasonable and worthwhile to draw conclusions from this experiment on the assumption that contamination is not responsible for the results. These conclusions must remain tentative and await confirmation or rejection from new and more satisfactory experiments designed to test their validity under more precise conditions.

The conclusions drawn from this experiment concern the process of induction and before discussing them in more detail, it is necessary to complete the discussion of the bearing of explantation experiments on the centrifugation problem.

Much of the indirect evidence against contamination is lacking in Pasteels' explantation experiment. The distinction between ectoderm and mesoderm has not the same resolution in *Rana temporaria* as in *Xenopus*. The time variant could not be considered by the nature of the results. No correlation between inductions in intact embryos and explants is given. The description of the explants does not suggest gross contamination. In another experiment, explants were made from early blastulae of *Rana*

temporaria, a stage rather insensitive to centrifugation, and these gave no inductions. Pieces of ectoderm transplanted to near the primary hind brain of neurulae, developed with neural tissue. The intact embryos did not produce inductions, and in this case Pasteels talks about a "subliminal" effect. This explant experiment affords some corroboration for the reliability of the techniques in so far as inductions did not occur in explants taken from embryos which were not destined to give secondary inductions. However, as contamination cannot be ruled out directly, and the onus is upon the experimenter to show that it does not occur, the verdict on Pasteels' experiment must be left open, as there is insufficient indirect evidence upon which to express a reasonable opinion.

To support the hypothesis that inductions in explants are caused by a stratification of cell contents, it is necessary to rule out contamination altogether. The stratification hypothesis is made very unlikely by Karasaki's and Yamada's explantation experiment, and by the introduction of the time variant in the author's experiment. In concluding that the secondary inductions from the blastocoele roof are caused by an inductive process, it does not really matter, in considering the author's experiment, whether the inductions in explants are due to a very low but significant level of contamination or not, when considering the origin of secondary inductions from the blastocoele roof of intact embryos. The only point in doubt is whether ectoderm alone forms the whole of the induction due to an inductive process or whether mesoderm is carried round adhering to the blastocoele roof and partakes in the formation of the mesodermal components. This is a purely academic argument. In the very smallest inductions on the posterior surface of the neurula, it is possible that the secondary axis is formed almost purely from ectoderm. In larger inductions from the blastocoele roof it is impossible to believe that quite large quantities of mesoderm are not involved. (See Descriptive section.) Therefore the conclusion reached concerning the secondary inductions developing from the blastocoele roof is that both the possibilities envisaged by Schechtman (page 3) take place.

At this point a relevant statement by Saxen and Toivonen (1955) might be quoted:

"wäre es nicht unmöglich zu denken, dass während der zentrifugierung und die untere Fläche der Epidermis-zellen aktivstoffe von der Geweben übergeführt wurden, gegen welche die Epidermis während der Zentrifugierung gepresst wird".

An induced origin for the secondary inductions from the blastocoele roof explains the general principle, that secondary inductions can be obtained from Anuran embryos but not Urodeles. Almost all the mesoderm of the Urodele late blastula lies on the surface of the embryo. After centrifugation and the obliteration of the blastocoele, contact between ectoderm and mesoderm is impossible where these two germ layers lie side by side as adjacent areas of the blastocoele roof. At least a part of the mesoderm of the Anuran late blastula does not lie on the surface of the embryo, but is distributed about the margin of the blastocoele floor. After centrifugation in these species, the blastocoele roof is pressed against the floor, with the possibility of ectoderm and mesoderm making contact. It is significant that all the mesoderm of the *Xenopus* late blastula lies internally, none lies on the surface of the embryo, and this species will produce the greatest range of accessory anomalies on centrifugation. On the other hand, *Discoglossus* embryos produce small, inconsiderable inductions, in a low frequency, and the fate maps for this species show a considerable area of mesoderm on the surface of the blastula.

The time taken for ectoderm to acquire a neural stimulus from adjacent mesoderm.

Apparently the first author to investigate this aspect of the induction problem was Marx (1925). He transplanted portions of the presumptive neural plate from embryos at different stages of gastrulation, beneath the ventral ectoderm of neurulae. He worked with *Triton alpestris*, *taeniatus*, and *cristatus*, the latter species providing the hosts. Ectoderm from early gastrulae did not induce; that from late gastrulae always did so; ectoderm from the large yolk plug stage sometimes induced.

Nieuwkoop (see review 1955) has suggested that the process of neural induction takes place in two stages. An archencephalic activation is followed by a rhombencephalic and spino-caudal transformation. Eyal-Giladi (1954) has produced good evidence for this

from her experiments on Axolotl embryos. Johnen (1956) has tried a direct estimation of the time required for induction by combining ectodermal sandwiches from *Triturus vulgaris* and Axolotl with anterior notochord from open neural plate stage, and removing the latter from the explant after a certain period of time. Her results support Nieuwkoop's two stage induction hypothesis and show that in Axolotl as short a period as five minutes allows an archencephalic stimulus to pass, whereas the transforming principle requires some ten hours contact in order to pass. In *Triturus*, activation requires a minimum of four hours contact, but transformation follows quickly afterwards.

In sharp contrast to these results are those from Table III, which shows that all regions of the nervous system appear at once in explants from *Xenopus* embryos made over ninety minutes after centrifugation. There seems to be no evidence for a temporal separation of the actions of an activating and a transforming principle.

Brahma (unpublished) has obtained similar results from ectodermal explants made from *Xenopus* embryos at various gastrulation stages. The first explants to give inductions are those taken from gastrulae a little while after the blastoporal ring is completed. These explants show spinal cord as well as brain inductions, which is strange because the inductions must have been mediated by head organiser i.e. prechordal plate (and doubtfully some presumptive anterior notochord), which in Urodeles gives only archencephalic inductions. On the basis of results like these one doubts the presence of regionally specific inductors in *Xenopus*.

Although these results seem in conflict with those predictable on Nieuwkoop's hypothesis, the explants from the centrifuged embryos show certain similarities to those made in an experiment by Takaya (19). Working with Urodeles he made explants from the open neural plate. From some of these he removed all the mesoderm, in others he removed only the notochord, and in the remainder he removed all the mesoderm except the notochord. Explants without any mesoderm developed large neural inductions; these consisted of solid masses of neural vesicles and forebrain with the occasional eye cup. Compare with the explants illustrated in Plate XI, figs. 1 and 2 and Plate XII. The large masses of neural vesicles and forebrain are on the sides of the explants away from

the mesoderm; next to the mesoderm the neural mass takes on a rhombencephalic character and auditory vesicles are present in all three explants. Those explants from Takaya's series which developed somite and no notochord, developed hind brain in cases where the proportion of mesoderm to neural tissue was low, and spinal cord when the ratio was high. Compare with the explants illustrated in Plate X and Plate XI, figs. 3 and 4. Note in particular Plate XI, figs. 3 and 4. In the upper half of the explant there is a spinal cord; in the lower half there is a solid neural mass (seen in serial sections to be much larger than the upper neuralization) which shows rhombencephalic characteristics. Thus the spino-caudal or archencephalic nature of the inductions is related to the presence or absence of adjacent mesoderm.

These relationships are well illustrated by part 3 of Table III. Three explants contain brain structures, two are without mesoderm. All the explants developing spinal cord contain somite. Six of the explants containing neural vesicles have developed without somite. Takaya concludes that the physical proximity of somite mesoderm causes the transformation of an originally archencephalic induction into a more posterior neural differentiation, and it seems best to apply the same conclusion to the explants from centrifuged embryos. By these considerations it seems likely therefore that discrete activating and transforming influences are at work during primary induction in *Xenopus*. Brahma however found no mesoderm in his explants bearing inductions made from mid-gastrula and later stages. Yet archencephalic and spino-caudal inductions occurred together in the earliest explants to induce. The conclusion must be that both activating and transforming principles act very quickly in *Xenopus* and in particular that although they may act in temporal succession, the one follows upon the other so quickly as to make them inseparable by explantation during gastrulation. The fact that spinal cord is always associated with somite in the explants from centrifuged embryos and that neural vesicles frequently occur when no somite is present, suggests that the transforming principle is weak or non-existent before the onset of gastrulation, for in these explants transformation has occurred as a response to the proximity of somite.

The origin of mesoderm in explants from centrifuged embryos.

It is worthwhile at this point to pass in review the known example of mesodermal induction. (See review by Spofford 1948.)

1. Implants of organiser into the blastocoele cavity can induce mesoderm in host ectoderm.
2. Ectoderm grafted on to the presumptive mesoderm of an early gastrula forms mesoderm after invagination.
3. Ectoderm transplanted into mesodermal regions of an embryo directly (without invagination) can form mesoderm.
4. The secondarily induced mesoderm possesses organising capabilities.
5. Certain non-living inductors induce mesoderm.
6. Ectoderm grafted on to the posterior region of the open neural plate partakes in the formation of tail somites.

Thus the induction of mesoderm from ectoderm is no strange thing. If one assumes that an inductive mechanism is responsible for the mesoderm found in explants from centrifuged embryos, why does not mesoderm occur in Brahma's explants from gastrulation stages? It is possible that during their development cells of the primary organiser change their inductive capacities. Some support for this idea comes from work by Okada and Takaya (1942). They showed that future head organiser of *Triturus pyrrhogaster* is a tail organiser before invagination, and only acquires head organising capacity afterwards. Later they modified this picture slightly on some results from explants experiments but the principle remains that the organising properties of a tissue may change during development.

One might imagine that the most primitive organising capacity a tissue could possess is the power of serial evocation of its like, and perhaps such a capacity is still present in some degree in presumptive mesoderm before gastrulation. This suggestion to account for the presence of a mesodermal induction in explants from centrifuged embryos would also explain the fact, discovered by ~~Benda~~ Pasteels, that in centrifuging certain species at the beginning of their sensitive period, their inductions tend to have a

large mesodermal component to a small or non-existent neural component, and that centrifugation at the end of the sensitive period reverses this relation.

Toivonen (1954) predicts that a mesodermal induction should take relatively longer to pass from inductor to ectoderm than the activating and transforming principles; the results from parts 2 and 3 of Table III suggest rather the opposite, but the numbers are not convincing enough to warrant any firm conclusion. The conclusion from this experiment is that the mesodermal inductor takes no longer to pass than the neural activating stimulus and may pass in a slightly shorter time.



SUMMARY

1. a) By investigating the immediate effects of centrifugation,
 b) by studying the further development of centrifuged embryos,
 and c) by retrogressive deductions from the character of the abnormalities produced by the treatment,
 three causes are suggested, working alone or together, to account for the various anomalies, defect and accessory, developing in *Xenopus* embryos centrifuged at the late blastula and early gastrula stages. They are:
 - i) An unspecific effect, the microcephalic syndrome, induced as a response to any injurious treatment. This is responsible for most of the defect anomalies.
 - ii) An induction in the collapsed blastocoele roof which becomes finally located on the ventral surface of the neurula. Trapped mesoderm cells are probably carried on to the ventral surface along with this ectoderm in all except the smallest inductions. The variety of inductions produced in this manner is described.
 - iii) A fragmentation of the axial individuation field due to mechanical hindrance to the morphogenetic movements. Slight and severe abnormalities produced in this way are described.
2. Efforts to produce similar inductions to those produced by centrifugation by imitating the initial effects of the treatment by other means have failed.
3. The proportion of inductions in a batch of eggs has been significantly changed by environmental influences. This was done as a test of Pasteels' theory of the origin of inductions through intra-cellular stratification.
4. a) A repeat of Pasteels' experiment, in which he made ectodermal explants from newly centrifuged embryos was made, and contrary to Pasteels' findings, an entirely negative result was obtained - explants developed no neural and mesodermal structures.
 b) The experiment was extended to include the time variant. Results:
 - i) Explants made ninety or more minutes after centrifugation produced neural and mesodermal structures; those made before ninety minutes did not.

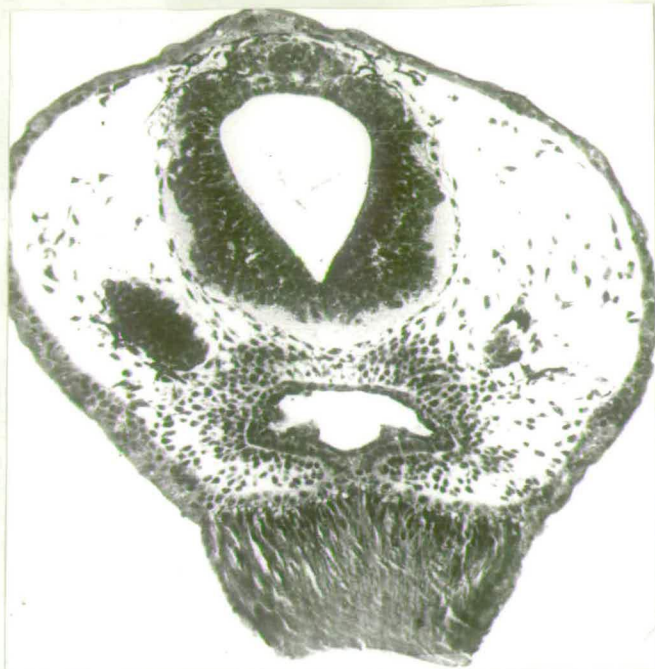
- ii) Where nervous tissue occurred without adjacent mesoderm, it took the form of neural vesicles, or when in proximity to somite the neural induction took the form of hindbrain or spinal cord.
5. The rationale of explant experiments is discussed, with particular reference to the possibility of mesodermal contamination through an unreliable technique. It is concluded that contamination is an unlikely cause of the above results and an inductive mechanism is preferred.
 6. It is concluded that the results from the explant experiment support clause 1. ii) above, and this is used to explain the fact that generally speaking Anurans do and Urodeles do not produce inductions when centrifuged.
 7. The results of the explant experiment are discussed in the light of Nieuwkoop's two-step inductions hypothesis. It is concluded that the transforming influence is weak before gastrulation and that the transformations in these explants occur as a response to the proximity of induced mesoderm. Ninety minutes therefore represents the activation time in *Xenopus* at 18°C.
 8. The presence of somite in the explants is explained as due to a mesodermal induction, from late blastula presumptive mesoderm cells, and partaking of the nature of a serial evocation. It is suggested that this represents a primitive stage in the ontogenetic development of the organiser.

PLATE I

Whole embryo control specimen. Head and anterior trunk region of a tadpole fixed 2 days after hatching.

- Fig. 1. Telencephalon, olfactory placodes, and adhesive gland.
Scale: $\times 1\frac{1}{2}$ the other figures.
- Fig. 2. Midbrain region, eyes and lens, optic nerve seen leaving eye cup on right, pharynx and pre-cartilage.
- Fig. 3. Section through the posterior level of the eyes, first signs of notochord.
- Fig. 4. Hindbrain, auditory vesicles, large pharynx, heart, notochord and small somites.
- Fig. 5. Anterior trunk region, pronephric tubules.

The scale of the explants is $2-2\frac{1}{2}$ times the magnification of the sections of whole embryos.

Fig. 1. ($\times 1\frac{1}{2}$)Fig. 2. ($\times 1$)Fig. 3. ($\times 1$) PLATE IFig. 4. ($\times 1$)Fig. 5. ($\times 1$)

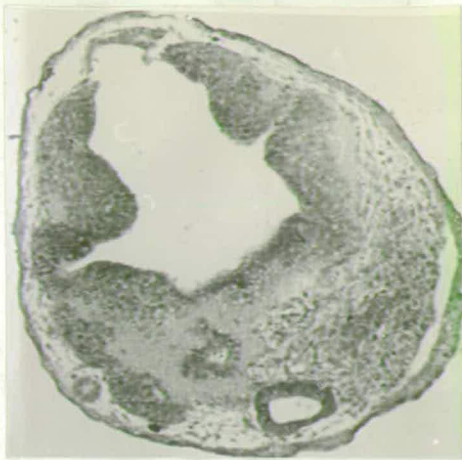


Fig.
1.



Fig.
2.

PLATE II

Embryo GC50-4. Centrifuged as late blastula at 670g for 4 minutes. Fixed 2 days after hatching. The embryo carries no induction and exhibits the microcephalic syndrome in a pure and severe form.

Fig. 1. Fore brain just posterior to adhesive gland and showing cavity of latter.

Fig. 2 A little posteriorly. Eyes absent, twin notochords, no pharynx.

Fig. 3. Hindbrain, single auditory vesicle, huge notochord, pharynx.

Fig. 4 Medulla, somites, pharynx, no pronephros or heart.
Trunk region and tail normal.

Fig. 3.

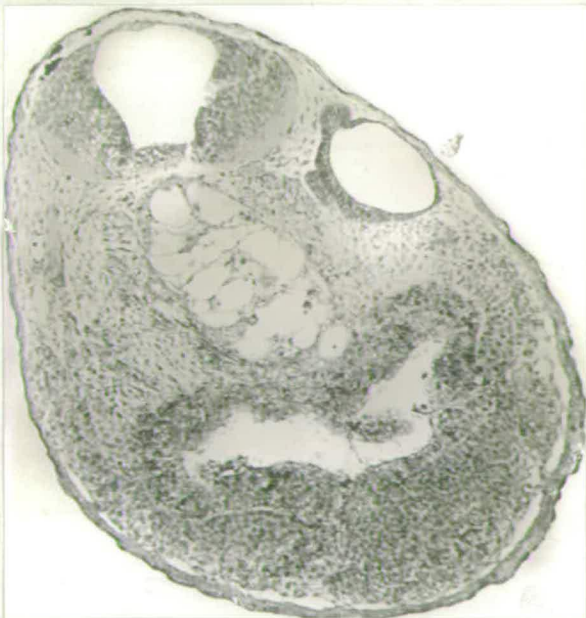


Fig. 4.



PLATE III

Embryo BC16-1 centrifuged as a late blastula at 480g for 4½ minutes. Fixed 2 days after hatching.

An example of the largest type of induction that arises from the blastocoele roofectoderm.

- Fig. 1. Prosencephalon doubled at level of adhesive gland.
- Fig. 2. Eyes associated with secondary axis, absent from primary axis. Notochord and somites present. No pharynx.
- Fig. 3. ~~Eye~~ Primary axis normal, anterior trunk level. Secondary hindbrain. No auditory capsules. Section filled with endoderm.
- Fig. 4. Mid to posterior region. Primary axis normal, secondary axis beginning to diverge from the midline into a secondary tail.

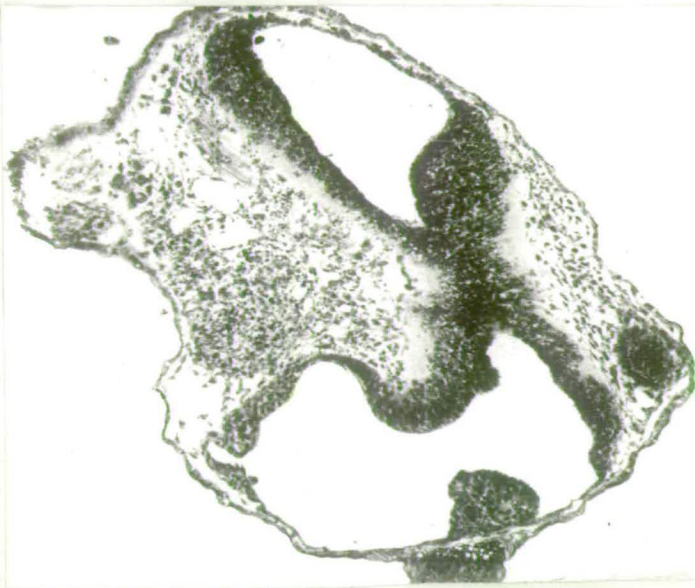
PLATE III

Fig. 1.

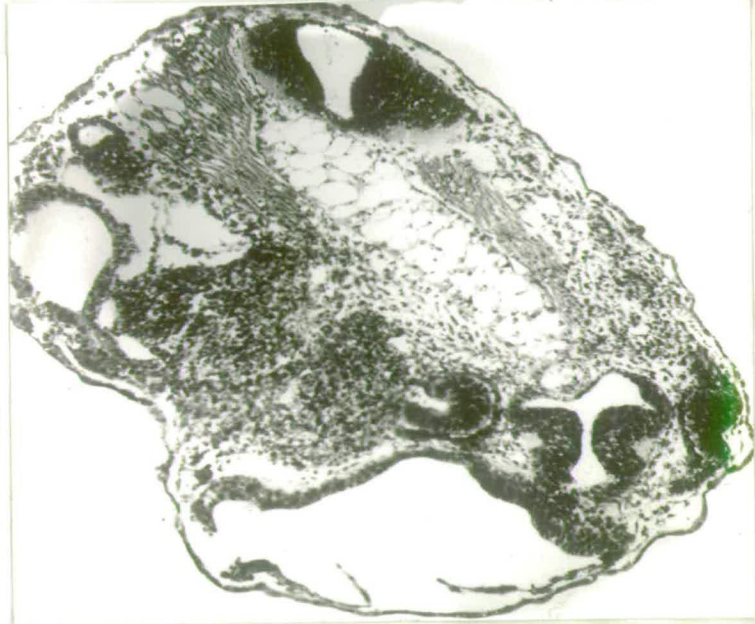


Fig. 2.

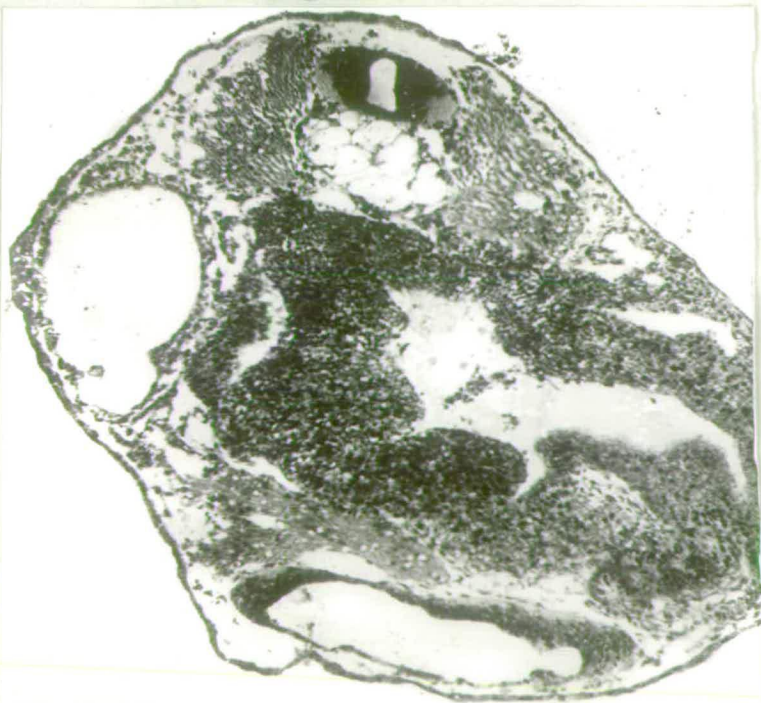


Fig. 3.

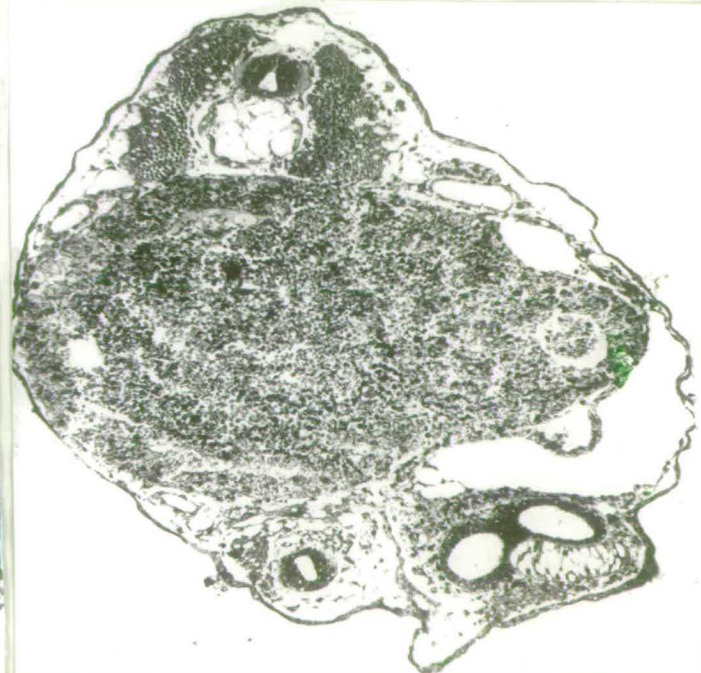


Fig. 4.



Fig. 1



Fig. 2

PLATE IV

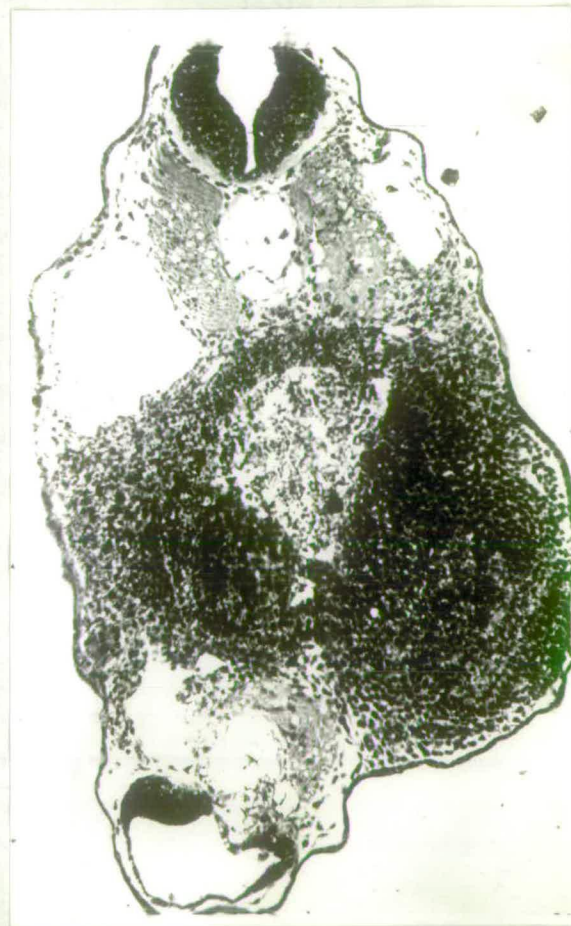
Fig. 3

Embryo BC15-3. Centrifuged as late blastula at 480g for $4\frac{1}{2}$ minutes. Fixed 2 days after hatching. A similar embryo to BC16-1 illustrated on Plate III.

Fig. 1. Adhesive gland mid-ventrally. Cavities of primary and secondary brains separate, eye tissue associated with secondary axis.

Fig. 2. A little posteriorly, large internal eye mass associated with secondary axis. Small auditory capsule near primary hind-brain. Notochord associated with each axis. No pharynx.

Fig. 3. Anterior trunk region. No heart, pharynx, pronephros.



BC 15-3

PLATE V

Embryo BC13-1. Centrifuged as late blastula at 480g for 4½ minutes. Fixed 2 days after hatching. A secondary axis from the blastocoele roof which runs into the primary hindbrain. The primary axis is very strongly microcephalic.

Fig. 1. Anterior extremity. Forebrain very small, without cavity, adhesive gland displaced laterally, section filled with pharyngeal tissue and includes a little nutritive endoderm ventrally.

Fig. 2. Probably midbrain region. Sense organs entirely absent. Brain very solid. Section two-thirds filled with pharyngeal tissue and nutritive endoderm.

Fig. 3. Left and right reversed. Hindbrain region. Secondary nerve cord running into primary hindbrain.

Fig. 4. Trunk region. Primary axis normal. Two axes quite separate. Secondary axis consists of a small spinal cord underlain by a somite block.



Fig. 1



Fig. 2

Fig. 3

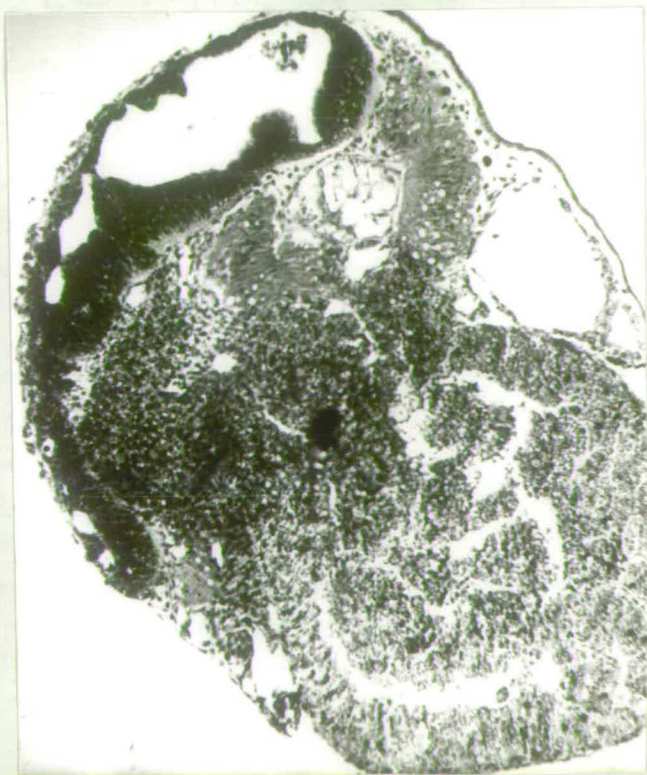


Fig. 4

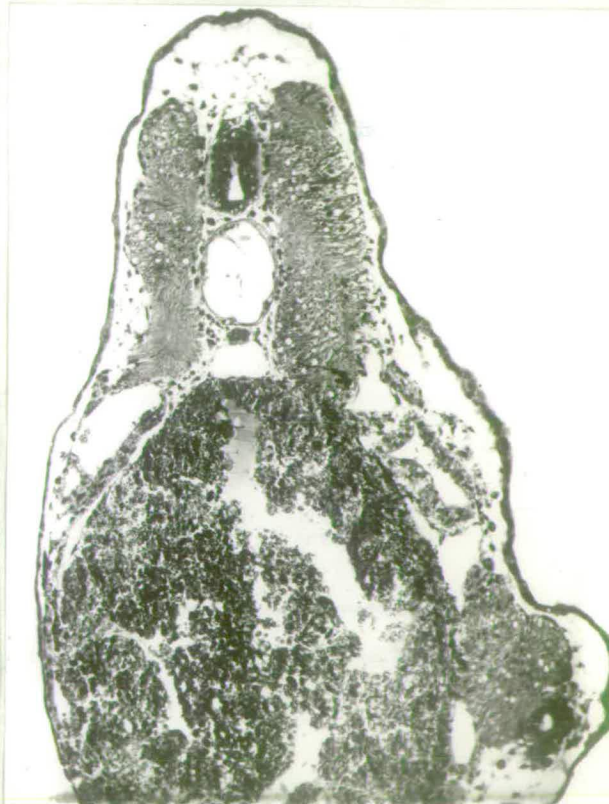


PLATE VI

Embryo BC16-3. Centrifuged as late blastula at 480g for $4\frac{1}{2}$ minutes. Fixed 2 days after hatching. An embryo exhibiting an induction that has arisen from a fragmentation of the mesodermal individuation field. This is not an extreme example.

Fig. 1. Some primary microcephaly. A single eye, well-formed notochord and somite, pharynx, adhesive gland displaced laterally.

Fig. 2. Hindbrain region, splitting into two nerve cords.

Fig. 3. Trunk region. Two spinal cords quite separate, three somites, centre one shared.

Fig. 4. Posterior trunk region. Secondary axis leading into a secondary tail.



Fig. 1 (x 1)



Fig. 2 (x 1)



Fig. 3 (x 1)

Fig. 4 (x $\frac{3}{4}$)

PLATE VII

Embryos GC50-7, 6 and 1. Centrifuged as late blastula at 680g for 4 minutes. Fixed 2 days after hatching.

- Fig. 1. GC50-7. Anterior trunk region. A duplication of the spinal cord covering about six sections (8). This is the only secondary manifestation in this embryo, which shows a fairly high degree of microcephaly. This is the lowest type of induction caused by "fragmentation" of the individuation field.
- Fig. 2. GC50-6. Head section. An extreme example of microcephaly. Very little nervous tissue induced.
- Figs. 3 & 4. An extreme example of an induction produced by a fragmentation of the individuation field. Two axes, the left bearing brain and the right spinal cord, quite separate, and lifted off the rest of the embryo. Posteriorly they sink into the yolk and unite.
- GC50-1

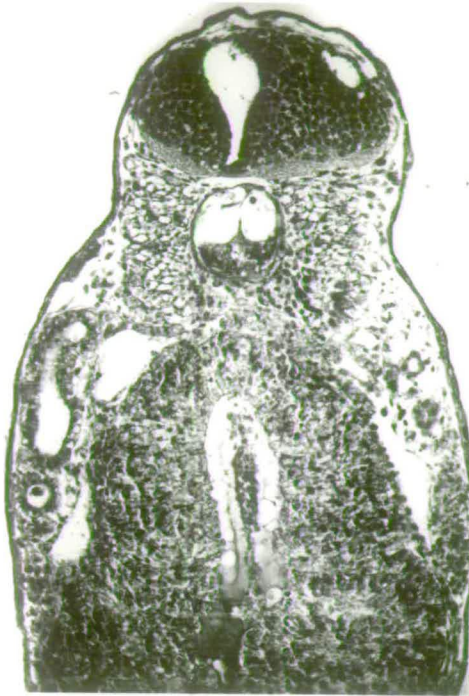


PLATE VII

Fig. 1

Fig. 2

Fig. 3

Fig. 4

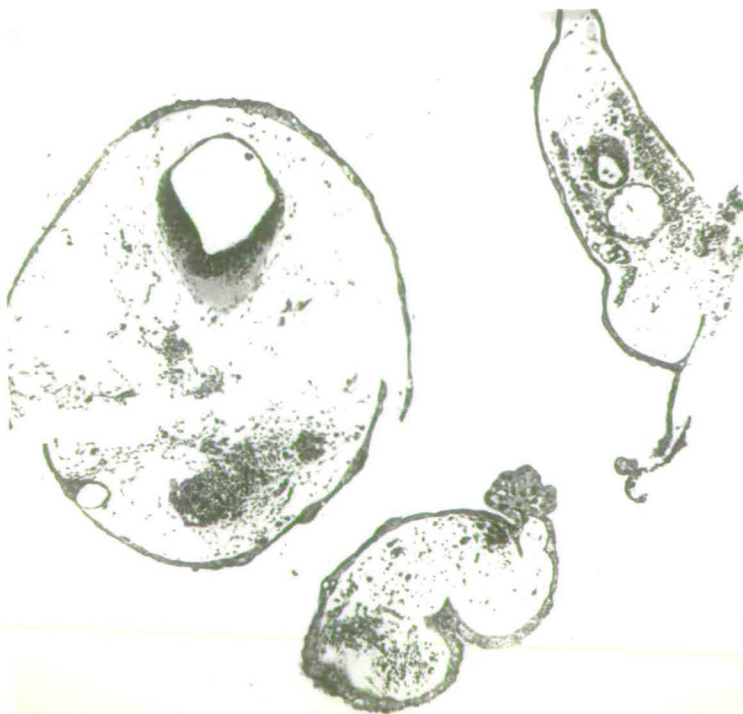


PLATE VIII

Embryo GC11-2. Centrifuged at 670 g for 5 minutes. Fixed about 1 day before hatching. Extreme example of inductions produced by a fragmentation of the individuation field.

- Fig. 1. Anteriorly. Two separate axes. One lifted off yolk, containing brain and chorda; the other containing a spinal cord.
- Fig. 2. Axes both on yolk mass. Two more small axes close together on ventral surface associated with some somite mesoderm.
- Fig. 3. More posteriorly. Two dorsal nerve cords uniting. Small ventral cords disappeared.
- Fig. 4. Posterior extremity. Nerve cord disappeared, common chorda and somites.

PLATE VIII

Fig. 1.



Fig. 2.

Fig. 3.



Fig. 4.



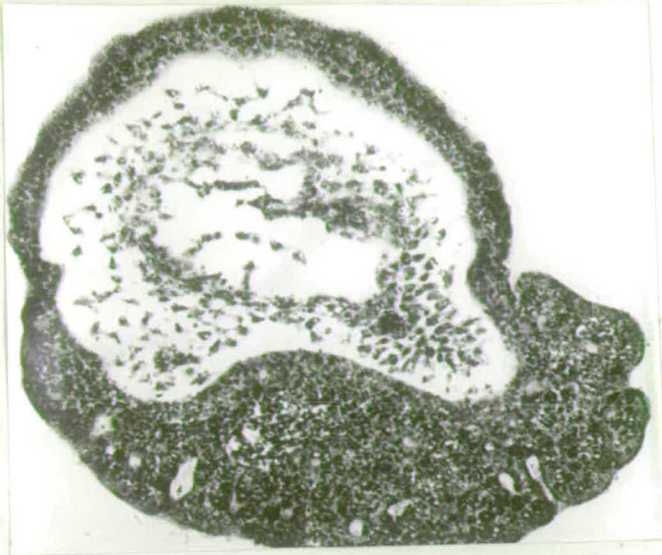
ECTODERMAL EXPLANTS FROM TABLE III

Fig. 1

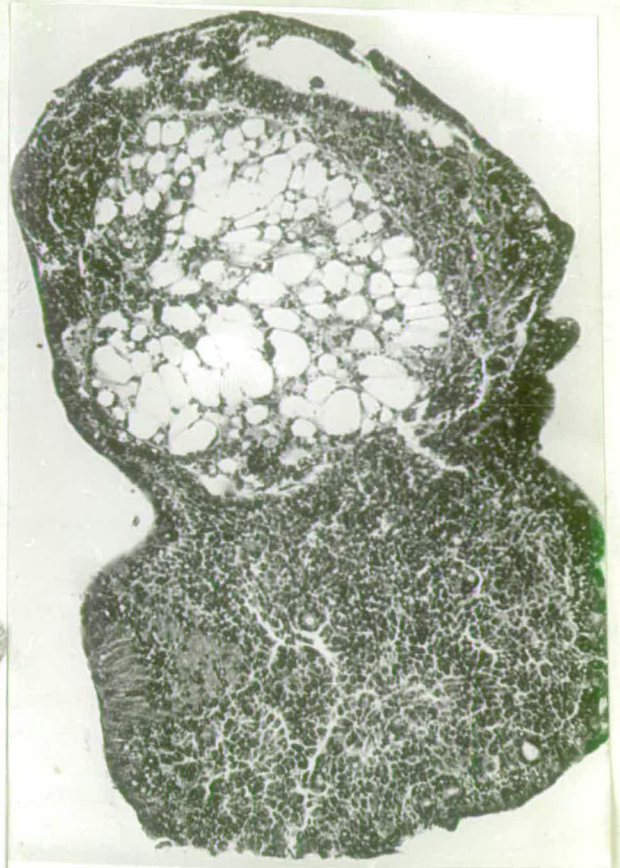


Fig. 2

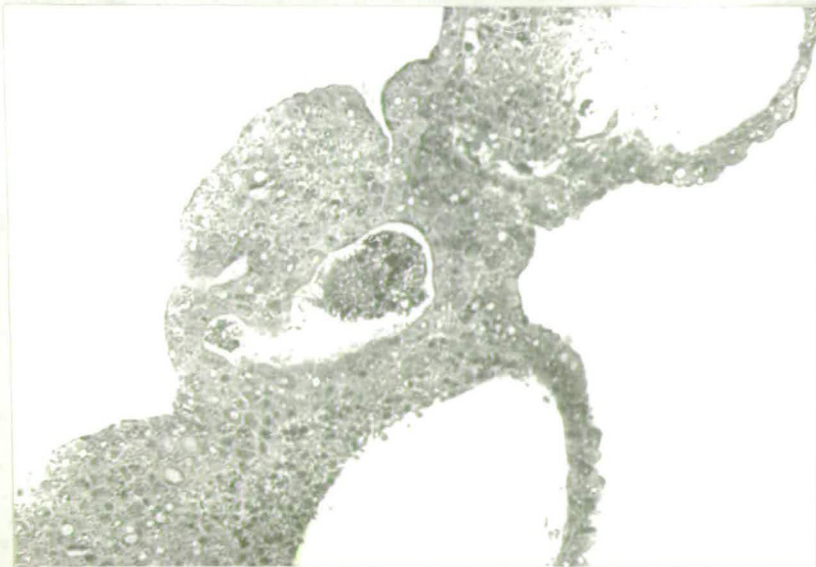


Fig. 3

PLATE IX

Fig. 1. D45-1. Control.

Note small lacunae in the thickened ectoderm.

Fig. 2. Large mass of notochord, also adhesive gland.

Fig. 3. D68-2. Contaminated explant. Small portion of endoderm or mesoderm in the centre of the picture.

ECTODERMAL EXPLANTS FROM TABLE IIIPLATE X

The formation of a primitive axis.

- Fig. 1 & 2. D55-2. Spinal cord underlain by somites, large block mesoderm on right. Typical fin formation top right, consisting of an ectodermal fold and mesenchyme.
- Fig. 3. D45-3. Spinal cord, possibly some brain tissue, underlain by notochord and somites.
- Fig. 4. D60-2. Very small axis with spinal cord, notochord and somites.
- Fig. 5. D45-2. Spinal cord, notochord, and well-formed line of very small somite blocks.
- D60-2

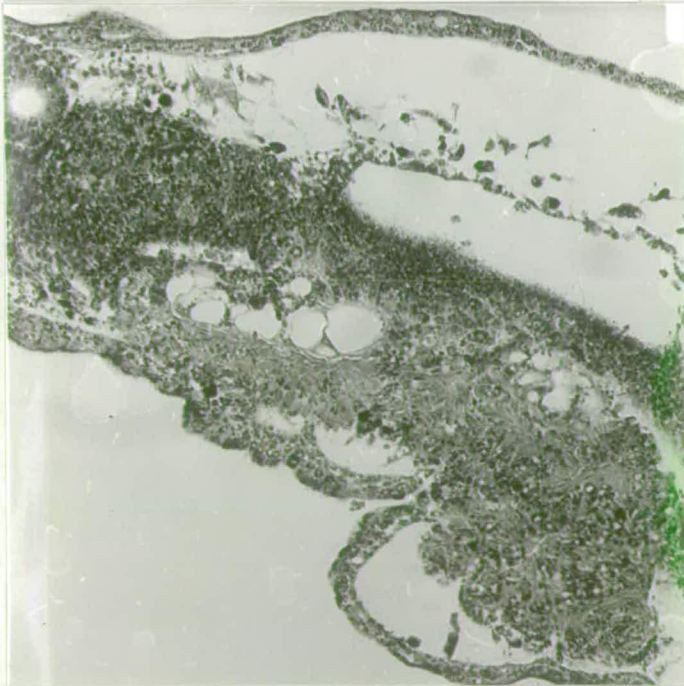
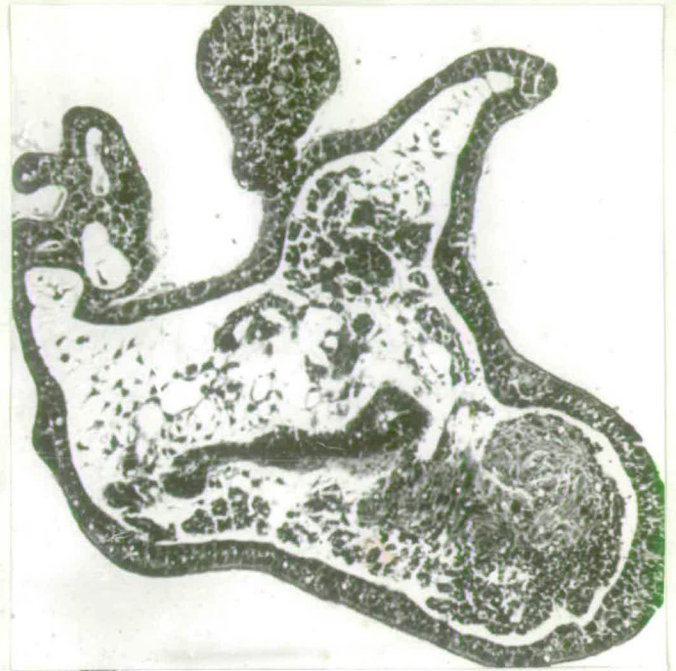
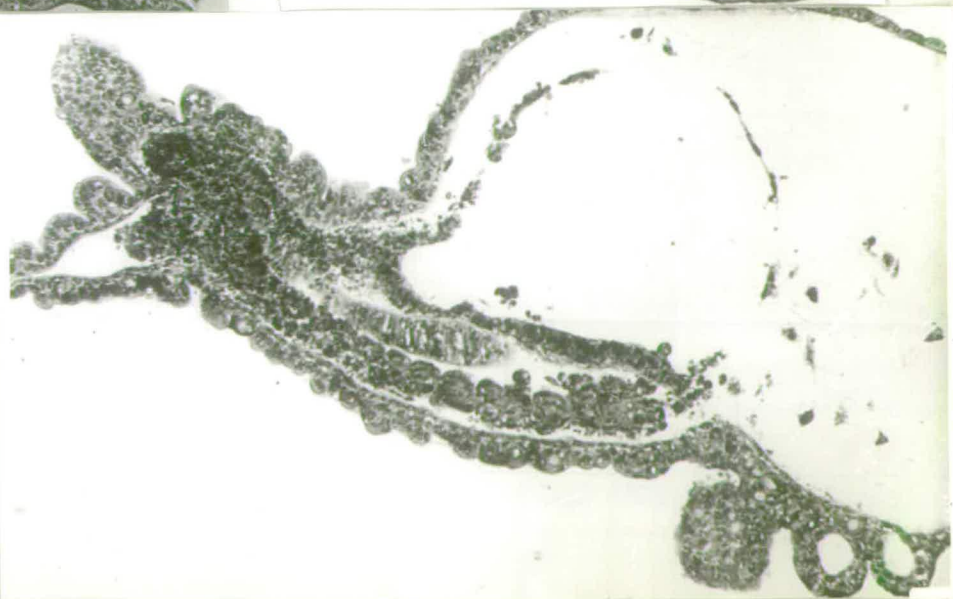


PLATE X

Fig. 1 Fig. 2

Fig. 3 Fig. 4

Fig. 5



ECTODERMAL EXPLANTS FROM TABLE IIIPLATE XI

Fig. 1. D61-2. Large central mass of neural vesicles. On the right a forebrain, on the left an auditory vesicle.

Fig. 2. Eye cup in centre of neural mass.

Figs. 3 & 4. D54-1. Spinal cord, notochord and mesenchyme in upper half of explant, neural vesicles beneath. Ectodermal excrescences.



Fig. 1



Fig. 2

PLATE XI

Fig. 3



Fig. 4

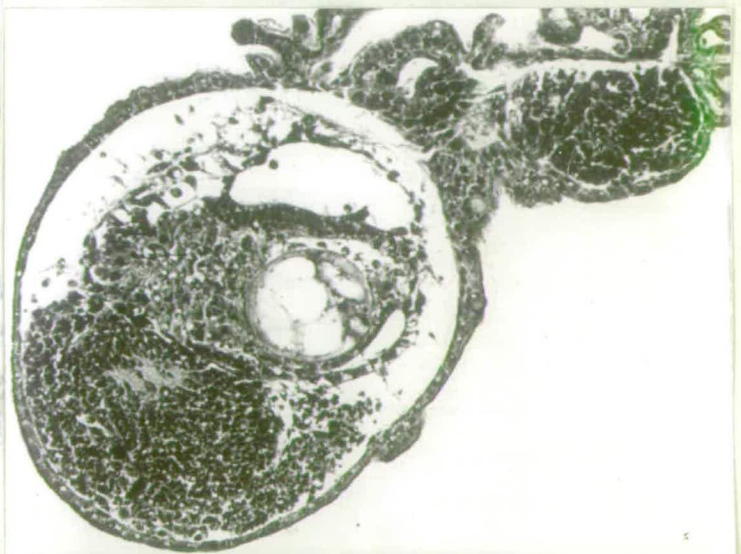


Fig. 1



Fig. 2



Fig. 3



PLATE XII

Fig. 1 & 2. D57-1. Adhesive gland. Large neural mass. Irregular optic cup top centre. Hindbrain on right, and auditory vesicles.

Fig. 3. D60-2. Neural vesicles and auditory capsule on left, notochord and somite on right.

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