

A New Technique for the Cultivation of the Chick Embryo *in vitro*

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WITH ONE PLATE

INTRODUCTION

THE inaccessibility of the chick embryo in the egg has led to the invention of a large number of *in vitro* techniques for its cultivation and study. The simplest of these techniques consists of little more than pouring the entire egg contents into a suitable container and taking precautions against excessive evaporation or bacterial infection (e.g. Assheton, 1896; Schmidt, 1937; Vollmar, 1935; Romanoff, 1943). Although these methods assist observation of gross changes occurring in the embryo or its membranes, they are of little use to the embryologist who wishes to make detailed observations or operations. It is very difficult to determine the outline of, for example, a primitive streak, whilst the blastoderm still lies on the yolk; and any operation on the blastoderm is usually followed by a slow leakage of yolk through the wound, which ruins the preparation.

The most successful method devised hitherto for explanting the blastoderm in isolation from the yolk is that of Waddington (1932). It is essentially an adaptation to chick blastoderm culture of a tissue culture technique of Fell & Robison (1929). The blastoderm is transferred to the surface of a clot of fowl plasma and embryo-extract maintained in a watch-glass in a moist chamber. In this position it is readily accessible to observation and manipulation. A modification has recently been introduced by Spratt (1947) who uses a clot stiffened by agar and containing diluted egg albumen or yolk. Except in a few details the two types of clot give the same result.

The Waddington technique has proved of great value to chick embryology. Nevertheless, it has considerable limitations. Primitive streak blastoderms usually develop only as far as the beginning of flexion and torsion—equivalent to about 30 hours' development *in ovo*. Embryos explanted at earlier stages rarely develop so long. A functional blood circulation is rarely established, and if it is death always occurs shortly afterwards; this is still the case even when the embryo has a functional circulation at the time of explantation. The rapid blastoderm expansion that occurs during the first few days *in ovo* is almost completely

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brought to a standstill after explantation to a clot surface. The embryos produced are stated to be abnormally small and slow-developing (Waddington, 1932).

The technique to be described here was designed to overcome some of these limitations. It consists essentially in supporting the explanted blastoderm on a piece of vitelline membrane stretched across a glass ring. The nutrient medium is fluid albumen. Blastoderms explanted at primitive streak stages continue expanding normally, and frequently produce large well-developed embryos with functional blood circulations. The technique is suitable for the explantation of blastoderms at all stages up to 48 hours of incubation.

METHOD

The whole explantation should be carried out under sterile conditions.

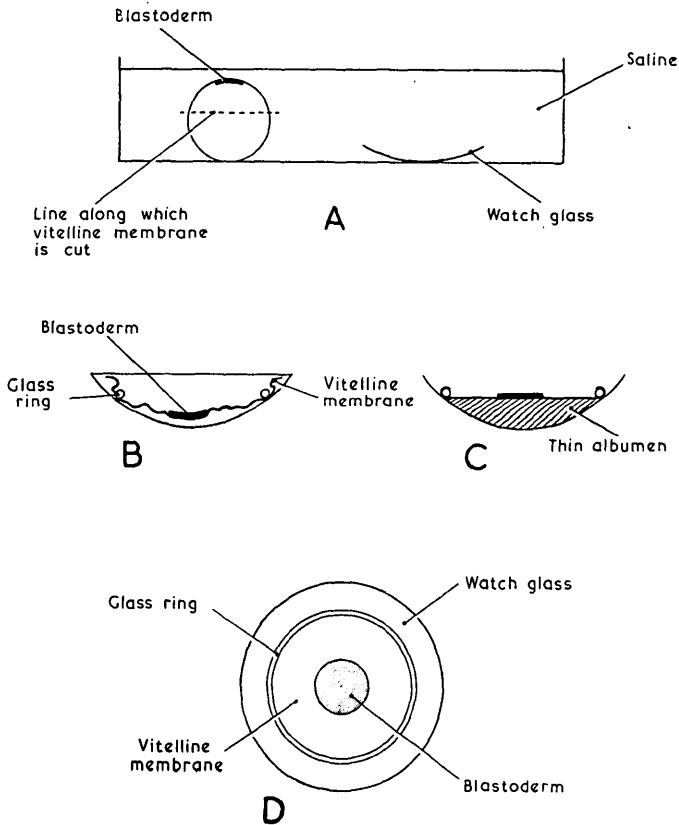
An egg at the required stage of development is opened by tapping round the broad end with the handle of a scalpel. The thick albumen is removed with blunt forceps, some of the thin albumen is poured into a container to be kept for later use, and the yolk and remaining albumen is poured into a wide dish containing sufficient Panett & Compton's saline to cover the yolk completely: 3.5 cm. is a convenient depth.

At this stage any thick albumen still adhering to the vitelline membrane must be removed with forceps. If the embryo is not already uppermost, the yolk is gently turned until the embryo is brought to that position. A watch-glass is placed in the dish ready to receive the blastoderm, and the vitelline membrane is then cut with scissors along a line passing slightly above the equator of the yolk (Text-fig. 1A). The cut edge of the membrane is seized with a pair of fine forceps in each hand and gently peeled off the surface of the yolk. With care the circle of membrane can be obtained almost completely free of yolk and with the blastoderm attached to it. The membrane is pulled through the saline to the watch-glass and placed in it with the blastoderm side uppermost. It is convenient to place the glass ring¹ on top of it at this stage as it keeps the membrane from drifting, and the watch-glass with contents can then safely be transferred to a Petri dish. The floor of the Petri dish is covered with water and cotton wool and acts, as in the clot techniques, as a moist chamber.

Subsequent stages are illustrated in Text-fig. 1 B-D. Saline is pipetted off until the upper part of the ring appears above the surface. The free edge of the vitelline membrane is folded inwards and gently pulled until the membrane forms a fairly flat surface. Care should be taken not to pull the membrane so tight that the blastoderm is distorted. Any small wrinkles left in the membrane at this stage can be ignored, as they tend to disappear shortly after the preparation has been returned to the incubator and the membrane then forms a perfectly smooth

¹ Rings of internal diameter 28 mm. and external diameter 33 mm. are a useful size. Larger rings can be used, but freeing correspondingly greater areas of vitelline membrane from the yolk introduces extra technical difficulties.

surface. The edge of the membrane can now be trimmed with scissors. All but a few drops of saline are removed from above it (the blastoderm should appear thoroughly moist but it is most important to avoid leaving a pool of saline over it). The saline under the membrane is replaced with thin albumen.



TEXT-FIG. 1. A. Stage preparatory to peeling off vitelline membrane and blastoderm. B. Contents of watch-glass after removal from dish of saline. C. Completed preparation. D. Completed preparation from above.

It sometimes happens that during the manipulation part of the periphery of the blastoderm becomes loosened from the vitelline membrane. It will readily reattach itself on further incubation provided it is pressed close against the membrane. To ensure this, the membrane surface should be left as dry as possible for a few hours until attachment has taken place, and then a few drops of saline may be added.

Very young blastoderms (pre-streak or early streak stages) are only lightly attached to the vitelline membrane and tend to be left behind if the latter is peeled off the yolk. To avoid this it is better in explanting young blastoderms only to free the edges of the circle of vitelline membrane from the yolk by pulling it, and then to use a spatula to lift off the remainder together with the blasto-

derm and a certain amount of underlying yolk. The yolk may subsequently be gently scraped away with small knives.

RESULTS

Survival rate

The proportion of normal blastoderms that survive this form of explantation is extremely high. Out of 149 explants (mainly definitive primitive streak or head-process stages) development was found to continue in 139—a survival rate of about 93 per cent. This figure is based on a number of experiments involving eggs laid at all seasons of the year.

Extent of embryonic development

Sixty-nine explanted blastoderms (definitive primitive streak or head-process stages) were incubated until embryonic development ceased. Sixty-six of them reached at least the 10-somite stage. Fifty-three developed a functional blood circulation and forty-four went on to show pronounced flexion and torsion. After this the mortality rate increased rapidly and only fourteen reached early limb-bud stages. None developed farther.

Expansion of the blastoderm

As far as can be judged from a superficial examination, the blastoderm expands normally. Both ectoderm and endoderm spread outwards evenly until they occupy the whole area enclosed by the ring. There are no obvious abnormalities as during growth on clots (e.g. formation of cysts). After the whole available area has been covered expansion ceases; there is no tendency to form folds.

The rate of expansion also appears to be about normal. As a standard of comparison a graph was used based on the diameter of 80 blastoderms incubated *in ovo* for lengths of time ranging from 8 hours to 48 hours.

Six blastoderms explanted between 19 and 25 hours of incubation were measured after a total incubation period of 39 hours. The blastoderm diameter then had approximately the same average value (17·4 mm.) as that determined from the graph of controls *in ovo*.

In another experiment two blastoderms were found just to have filled rings of internal diameter 28 mm. after 47·5 hours' incubation (of which 23·5 hours were *in vitro*); again this agreed well with the controls.

Rate of embryonic development and size of embryo

Average values of growth rates of explanted embryos were compared with the data given by Hamburger & Hamilton (1951) for normal growth *in ovo*. The time taken for definitive primitive streak blastoderms to develop 20-somite embryos was approximately the same (about 32 hours) *in vitro* as *in ovo*. After the 20-somite stage development *in vitro* became progressively slower than *in ovo* and ceased altogether at about 30 somites.

Insufficient measurements have been made to give a detailed comparison between growth in size of embryos explanted by this technique as compared with those *in ovo*. However, blastoderms explanted at primitive streak stages usually give 12-somite embryos about 6 mm. long, and 30-somite embryos about 9–10 mm. long, which appears to be approximately normal.

Amnion development

The development of the amnion seems to be markedly affected by explantation. It has frequently been observed that the amnion is less developed than expected for an embryo of a given stage. Several embryos that developed as far as limb bud formation were found to have no trace of an amnion although, according to Hamburger & Hamilton, it should by this stage almost have covered the embryo. Sometimes, however, the amnion does partially develop; in some explants it has been found covering about half the embryo.

DISCUSSION

The results indicate that this method of blastoderm explantation has considerable advantages over the previous methods involving explantation on to clot surfaces. The survival rate is higher and the amount of embryonic development that can be expected is greater. Thus primitive streak blastoderms frequently develop embryos with functional blood vascular systems and showing flexion and torsion—a rare occurrence on clot surfaces. The blastoderm expands normally until the end of the second day of incubation, and, judging by the growth rate and size of the embryo, the path of embryonic development is probably nearer the normal in these explants than in those on clots.

However, although a much higher proportion of embryos develop to advanced stages by this method as compared with clot techniques, the most advanced stage of development that can be reached appears to be about the same in both cases. Death inevitably occurs shortly after the start of limb-bud formation. Hughes (1935) suggests that the blood-vessels of embryos on clot surfaces become blocked because multiplication of corpuscles takes place at the normal rate, whilst the vessels themselves fail to increase in size. Possibly similar events occur in blastoderms growing on vitelline membranes, but it seems likely that other mechanisms also contribute towards the death of the embryo. The inverted position of the embryos may produce serious mechanical interferences with development after a certain stage; certainly this seems a likely explanation for the abnormal amnion development. The pool of watery fluid that accumulates above the blastoderm after 2 days of incubation *in vitro* may interfere with respiratory exchanges between the area vasculosa and the atmosphere; Spratt (1950) has shown that early chick development is very sensitive to any restriction in oxygen supply. Or it may be that the nutrient medium used is inadequate to support development beyond a certain stage.

The explantation technique that has been described here appears to be well



suiting to studies of early chick embryology involving micro-operations. It has also been used to investigate the mechanism of blastoderm expansion and the transfer of fluids through the blastoderm. These two subjects will be discussed in subsequent publications.

SUMMARY

1. A new technique is described for the cultivation of chick embryos *in vitro*. It consists essentially in supporting the explanted blastoderm on a piece of vitelline membrane stretched across a glass ring. The nutrient medium is fluid albumen.

2. Primitive streak blastoderms explanted by this technique: (a) frequently develop as far as the formation of a functional blood circulation, together with flexion and torsion of the embryo; (b) show normal expansion across the vitelline membrane; (c) develop at a normal rate until after the 20-somite stage.

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EXPLANATION OF PLATE

- A. Blastoderm explanted at head-process stage. Incubated 43 hours *in vitro*.
 B. Blastoderm explanted at definitive-primitive-streak stage. Incubated 43 hours *in vitro*.

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