Special Problems of Experimenting *in ovo* on the Early Chick Embryo, and a Solution

by P. H. S. SILVER[1]

From the Department of Anatomy, Middlesex Hospital Medical School, London

INTRODUCTION

IT seems to be generally accepted that experimenting *in ovo* on the chick during the early stages of development (up to about 48 hours) is fraught with the greatest difficulty. After about this time no serious technical problems arise and a high proportion of successful results can be expected. It is natural to ask why there should be this change-over from extreme difficulty to reasonable simplicity. New (1955) attributed to this 'inaccessibility of the chick embryo in the egg' the invention of his own and many other *in vitro* methods during the last 30 years. There is no doubt that, when short-term experiments only are required, *in vitro* methods will probably always be preferred. But all *in vitro* methods suffer from the disadvantage that the embryo cannot be expected to survive for more than 48 hours or so after explantation. There are many experiments, however, in which operative interference is required at stages of up to about 12 somites, and in which it is necessary for the embryo to develop thereafter for a considerably longer time.

Grabowski (1956) gives some important technical details for operating *in ovo*. His method depends upon two main steps: (1) only a very small hole, 0·1–0·2 mm. in diameter, is made in the vitelline membrane, and (2) the air space overlying the blastoderm is topped up with saline or watery albumen before replacing the window in the shell. This step, i.e. topping up, he describes as 'absolutely essential' to prevent drying of the exposed surface of the blastoderm. The egg is now rolled so that the blastoderm with its overlying vitelline membrane is faced with intact shell membrane. This is a very satisfactory method, but suffers from the disadvantage that, for many purposes, a hole of this size in the vitelline membrane is prohibitively small. It is extremely difficult to do much with a needle through the hole without tearing the membrane (which even when stained with Nile blue sulphate quickly loses the stain and is difficult to see) and thus accidentally enlarging the opening. Furthermore, if a

[1] *Author's address*: Department of Anatomy, Middlesex Hospital Medical School, Mortimer Street, London, W. 1.

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large extirpation is undertaken, it may not be possible to extract the fragment through such a small hole. One of the primary aims of the present investigation has therefore been to find a means of making a large opening in the vitelline membrane without prejudice to the embryo's survival.

In a previous communication (Silver, 1959) the role of the vitelline membrane in the production of abnormalities affecting the embryo and of the amnion was described. One of the main causes of trouble was the drying of the vitelline membrane with subsequent sticking to the embryo, or to its membranes, or to the expanding blastoderm. The mechanism of surface drying was thought to be related to the secretion of sub-blastodermal fluid, for New (1956) has shown that the blastoderm secretes fluid downwards into the yolk. This fluid is drawn from the albumen which overlies the blastoderm. Evidently it is this layer of albumen which is normally responsible for keeping the vitelline membrane moist, but even in the unoperated egg it is extremely thin. The point which arises from New's work, and which needs special emphasis here, is that we are dealing with a continuous process. Thus it will not be sufficient to prevent drying only while the actual experiment is being performed; dehydration of the supra-blastodermal fluid will continue during the later stages of development. What is needed is a method of replenishing the suprablastodermal fluid with fresh albumen *after* the egg has been returned to the incubator. 'Topping up' achieves this end but is inapplicable in the presence of a large opening in the vitelline membrane, for reasons which will be explained below.

When the normal experimental procedure is adopted, as soon as the window is cut in the shell membrane the blastoderm drops down, an air space appears, and, instead of being convex upwards as it normally is, the blastoderm becomes concave upwards. Under these conditions it is now possible to make a really large opening in the membrane exposing the whole of the area pellucida, without any tendency for herniation to occur, provided the embryo is orientated immediately below the centre of the shell window. If now, to prevent subsequent drying, we employ the Grabowski method and top up the egg, we find that as soon as the blastoderm regains its normal shape, becoming convex upwards once more, the embryo and surrounding blastoderm begin to spill out through the opening in the vitelline membrane.

We now seem to have reached an impasse. If we make an opening in the vitelline membrane large enough to permit major operative procedures, herniation will occur when we top up. If we do not top up, on the other hand, surface drying will ensue and adhesion between the embryo or its membranes and the vitelline membrane will take place, and the expansion of the area vasculosa will be arrested or very seriously retarded.

Silver (1957) devised one method of escaping from this difficult position by repairing the opening in the vitelline membrane with a single silk thread. This method suffers from the disadvantage, however, of being somewhat laborious; it may take up to 15 minutes. In fact, it is technically much more difficult than

experimenting on the embryo itself. A search was therefore undertaken to find a new technique capable of preventing drying without topping up.

In all, about 1,500 investigations have been carried out. It is not proposed to describe more than a small fraction of these, i.e. those which seem immediately relevant to the final method.

METHODS

Operating incubator

These experiments proceeded in a specially constructed operating incubator, connected to a suitable air-conditioning plant, in which the temperature was maintained at 99·5° F. and the humidity as near 100 per cent. as possible in order to prevent evaporation during the actual experiment. It was possible to observe the embryo for long periods, see what really did go wrong, and devise appropriate preventive measures. This apparatus was made of transparent perspex with double walls within which, in the roof, warm water circulated to prevent condensation. The microscope was excluded completely from the humid atmosphere. The platform of the microscope revolved around a vertical spindle in the manner of a turntable. Small button magnets were let into it and the eggs placed in an iron-bottomed dish, which adhered firmly to the turntable.

The apparatus was focused by a simple device operated with the feet, capable of raising or lowering the platform of the microscope. The vertical spindle of the turntable passed through the bench and rested on the end of a screw (point upwards). This screw was connected by a flexible cable to a reversible d.c. motor which was controlled with two micro-switches on the floor. The motor had a brake so that, when the current was turned off, it would stop dead without overrunning. With this set-up it was a great advantage to have both hands free at all times.

The observations reported here were based on experiments on White Leghorns. But investigations with other breeds and with ducks were also undertaken. The chick embryos were incubated for 30 hours prior to experimentation.

RESULTS

Observations in the operating incubator

Normal development, for at least 12 hours, would proceed in spite of a very large opening in the vitelline membrane, i.e. one exposing the whole area pellucida. In spite of the high humidity, surface drying still occurred, and evaporation was not, therefore, the causal factor. Clearly, one could not rewet by adding drops of saline or albumen, because in such a long-term procedure topping up with its consequent herniation would be certain to occur sooner or later as one would be adding to the total fluid content of the egg. Instead, the egg was twisted by hand clockwise and anticlockwise on its turntable; when

this was done the yolk, owing to its inertia, was seen to be in more or less constant movement in relation to the shell membrane. This constant movement of the vitelline membrane in relation to the inner surface of the shell membrane wetted it in a manner similar to the action of the eyelid in keeping the cornea moist. Furthermore, it was noticeable, as the egg was twisted first one way and then the other, that the relationship of the blastoderm and vitelline membrane also changed constantly, an additional factor militating against adhesion formation. These observations showed that, so long as the egg was kept in motion in the manner described, the exposed surface of the vitelline membrane remained wet and normal development of the embryo proceeded.

Results with turntable incubator

The next step in the inquiry was to place in the normal incubator a series of mechanically operated horizontal turntables (one for each egg) which rotated clockwise and anticlockwise around a vertical axis through an angle of about 90°, at a speed which could be varied from about 15 cycles per minute downwards. The aim was to reproduce mechanically those movements previously carried out by hand. Windows were cut in the shell and shell membrane in the usual way, and an opening of not less than 0·75 mm. was made in the vitelline membrane. Once the experimental procedure had been completed, the egg was not topped up, the shell window was refixed, and the egg placed on a turntable in the incubator so that the window exactly overlay the centre of the turntable. A speed of 8–9 cycles per minute was found to be the most satisfactory. Of 18 such preliminary experiments, 18 embryos reached stage 19 (Hamburger & Hamilton, 1951), i.e. the stage by which the amnion is completed. Of these embryos, 14 reached hatching. Of a control series of 36 embryos which had been placed in the same incubator but not on the turntables, only 2 reached stage 19, but even these were abnormal in that the area vasculosa was inadequately developed and the amnion was defective; neither survived the 7th day. The turntable incubator was used for a total incubation time of 72 hours, when it was found that the blastoderm usually came to the top with a facility which exceeded that of normal eggs at the same stage, when the eggs were rolled.

Experiments of various kinds concerned with head morphogenesis, all involving major operative procedures, have been performed with this method on embryos ranging from 4 to 12 somites. In all, 120 have been undertaken so far. Most of these experiments were terminated after 8–10 days, but many proceeded for much longer and some to hatching. Of these 120 experiments, 31 were unsuccessful, resulting either in death or in abnormality of the embryo or its membranes. It is very remarkable, however, that only 3 of these 120 embryos failed to reach stage 19. There is no doubt that this technique of incubation has provided one solution of the drying problem.

These results suggest that by this method, given adequate preliminary experience, it is possible to carry out operations during the early somite stages

with the expectation of a success rate comparable to that achieved when the experiments are performed very much later—e.g. for intracoelomic grafting.

Dangers of the method

The speed of rotation on the turntable is limited by the size of the opening in the vitelline membrane. If no opening is made here normal development will occur with speeds up to 15 cycles per minute. On the other hand, if a large opening is made in the vitelline membrane the speed should be as slow as possible, but it must be sufficient to maintain constant movement between the yolk membrane and the shell.

It is very important that the window in the shell membrane should be cut exactly in the right place directly over the embryo. If this has not been done, and the egg has to be tilted to bring the embryo to the central position, it is the author's practice to discard that egg and start again. If the blastoderm becomes eccentric after the experiment has been performed, then it is worth incubating, but a higher failure rate occurs. In fact, of the 31 failures reported above, 24 were described at the time of operation as 'very eccentric' and were therefore given a bad prognosis. But all others similarly described appeared to develop normally. In the remaining seven unsuccessful cases the cause of abnormality was not established.

The relation of the blastoderm to the shell membrane and the way it varies in its behaviour with differently shaped eggs are factors which must be borne in mind when selecting the eggs for experimentation. Eggs in which the blastoderm is not quite at the highest point of the shell, or is too near the blunt end of the egg, should be avoided. The object is to use eggs in which the blastoderm will remain central in position when the egg is rotating.

The only peculiarity which has been noticed is that the head and neck of the embryo begin to fall away from the surface level of the blastoderm into the yolk on the 4th instead of on the 5th day. But this often occurs after intracoelomic grafting and is presumably due to the presence of the air space and not a result of the use of a turntable.

Definitive method

1. Candle the egg and cut window in shell over the blastoderm *c.* 0·5–0·75 cm. square.
2. Place the egg in the operating incubator at temperature of 99·5° F. and nearly 100 per cent. humidity.
3. Candle the egg again and cut window in shell membrane exactly over the embryo.
4. Make opening in vitelline membrane as small as reasonable for experiment required. The position of the opening—over the embryo or to the side—is not important; sticking to the edge of the opening (Silver, 1959) will not occur as long as the vitelline membrane remains wet.

5. Twist the egg on the turntable under the microscope at frequent intervals during the experiment and, finally, as an extra precaution, rewet the whole of the exposed surface, not merely the embryo, with a glass rod or forceps by drawing the peripheral albumen towards the centre, so that it forms an unbroken film. It is now safe to replace and seal the shell window with wax. (The use of adhesive tape or wax paper should in my opinion be avoided because, whenever the incubator door is opened, dew immediately appears on the undersurface and this moisture must come from the suprablastodermal fluid.)
6. Replace the egg in the incubator on its own mechanical turntable (90° turn, alternately clockwise and anticlockwise, 8 cycles per minute) with the window uppermost and immediately over the central vertical spindle of the table. In theory the centrifugal forces acting on the yolk should then balance each other. Each egg should be left on its turntable until a total incubation time of at least 72 hours has elapsed. By this time the amnion is complete and the circulation well established and anti-drying measures are no longer required.
7. It is thought to be inadvisable to roll the eggs during the subsequent weeks of incubation. It is easy to roll 180° immediately after removing the egg from the turntable, but later the albumen, as it becomes viscid, will stick to the window. Once this has happened further attempts to roll the egg will only produce distortion of its contents.

SUMMARY

A method has been described for experimenting on the early chick embryo. By its use it is possible to make an opening in the vitelline membrane large enough for the easy performance of any major operative procedure. The air space over the blastoderm is not topped up. The egg is incubated on a horizontal turntable, rotating around a vertical axis, at about 8 cycles per minute through an angle of about 90°, until the embryo has reached the end of the 3rd day of development. Successful results, using embryos of 4–12 somites, have been achieved in 74 per cent. of 120 major experimental procedures. The rationale of the method has been explained.

RÉSUMÉ

Problèmes spéciaux à envisager pour opérer in ovo *le jeune embryon de poulet, et une solution*

Une méthode est décrite pour opérer le jeune embryon de poulet. Par son emploi, il est possible de faire dans la membrane vitelline une ouverture assez grande pour réaliser facilement n'importe quelle opération importante.

On referme la fenêtre de la coquille, sans emplir de liquide l'espace, contenant de l'air, situé au-dessus de l'embryon.

L'œuf est incubé sur une table horizontale tournant environ 8 fois par minute autour d'un axe vertical et d'un angle d'environ 90 degrés, et cela jusqu'à ce que la fin du troisième jour de développement ait été atteinte. Des opérations graves ont été ainsi réalisées avec succès sur des embryons de 4 à 12 somites dans 74 pour cent des essais effectués. La justification rationnelle de la méthode a été donnée.

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