Experiments on the Diffusibility of the Amphibian Evocator

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WITH ONE PLATE

INTRODUCTION

The discovery by Bautzmann, Holtfreter, Spemann, & Mangold (1932) that amphibian organizer material can still cause an induction after being killed gave rise naturally to the hypothesis that the active principle is a chemical substance which passes from the evocating graft into the reacting tissues. It has, however, remained very difficult to demonstrate that any such passage of material occurs. In this first joint communication, Mangold claimed that when pieces of living blastopore lip are left for some time in contact with an agar jel, the latter becomes inducing, presumably owing to the diffusion into it of an active substance. It has, however, not proved possible to repeat this observation. Most later authors have been impressed with the fact that induction nearly invariably fails if an organizer graft is not in immediate cell-to-cell contact with the reactive tissues, and have concluded that the active principle cannot diffuse through watery solutions. Holtfreter (1933) further showed that induction is prevented if a thin sheet of vitelline membrane is placed between graft and host tissues. Since the permeability of the vitelline membrane is not fully known, it is impossible to draw precise conclusions from this, but it would certainly seem to indicate that the active evocator is not an easily diffusible small molecule.

More recently, several reports have appeared which suggested that the diffusibility of the evocator through membranes might be worth re-investigation. Brachet & de Scoeux (1949) observed induction through a membrane with average pore size of 3-4 μ, but Brachet (1950) later concluded that this was probably due to an unspecific irritation of the reacting cells by the membrane itself. On the other hand, several workers with adult inducers have presented evidence which is suggestive of a diffusibility of active evocating substances. Holtfreter (1955) reported cases of induction by kidney tissue which was coated with agar. Several of the investigators who are attempting to analyse the nature of the heterologous inducers have suggested that compounds such as proteins, nucleo-proteins, or even nucleotides may be involved (see the review by von Woell-

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warth, 1956), and these might well be diffusible. Again, induction is known to be possible with liquid 'embryo extract' and solutions of certain carcinogenic compounds. But perhaps the most pressing reasons for re-opening the question arise from the recent works of Niu and Twitty (1953) and Grobstein (1954). The former have shown that if organizer material from an amphibian gastrula is allowed to age for some days in a suitable culture medium, it gives out into solution a substance or substances which are capable of inducing the formation of neural and other tissues in fragments of competent ectoderm. It does not follow that similar substances are emitted from organizer tissue at the time at which it is normally active in the young gastrula, but that possibility certainly exists. Grobstein demonstrated a clear-cut action of mesenchyme on epithelium from which it was separated by a porous filter. The materials used were of the mouse; and the 'inductive' action was perhaps more in the nature of a morphogenetic stimulus than the determination of a particular histogenetic trend; but again the results would seem to make it desirable to re-examine the possibility that the active principle of the primary amphibian evocator might be diffusible through a suitable membrane. Three types of experiment have been made with this aim in view, Gradocol membranes (obtained from the Wright-Fleming Institute, St. Mary's Hospital, London) being placed between the inducing and reacting tissues in various arrangements.

MATERIALS AND METHODS

Sterile conditions were maintained during and after the operations. Full strength Holtfreter solution, buffered at pH 7 (Deuchar, 1953), with 15,000 i.u./l. of both penicillin and streptomycin was used. Operated specimens, when completely healed, were transferred to 1/10 Holtfreter's solution containing 0·1 g./litre of sulphadiazine. Embryos were thoroughly washed with full strength Holtfreter's solution before the operations.

Operations were performed with fine tungsten needles, and the operating dishes were floored with 2 per cent. agar in which a small groove was made to hold the embryos in position during the operations. On occasions, thin glass bridges made out of coverslips of 0·15 mm. thickness were used to assist healing.

Embryos were fixed either with Smith or with Bouin, dehydrated through alcohols, and cleared in terpineol. They were embedded in paraffin, sectioned at 10 μ, and stained with celestine blue eosin.

Series A

The membrane had an average pore diameter (A.P.D.) of 4 mμ, and thickness 0·031 mm. Presumptive neural plate ectoderm of early crescent gastrulae of axolotl was cut near the region of invagination. It was lifted up gently and any cells which remained loosely attached to the inner layer removed carefully without injuring the tissue. A small rectangular piece of membrane was placed between the invaginating mesodermal sheet and the presumptive neural plate.
The presumptive neural plate was brought back to its original position. The embryos were left undisturbed till they healed completely, and then transferred to 1/10 Holtfreter and allowed to develop to the required stages. Six specimens were studied in this series.

**Series B**

The same membrane was used as in the series A, the experimental material being early gastrulae and early neural plate stages of *Xenopus laevis*. A square piece was removed from an early neural plate without damaging the underlying mesodermal sheet. Presumptive ectoderm of size similar to that of the tissue removed was excised from an early gastrula and grafted over the host with the membrane in between. After healing, the embryos were transferred to 1/10 Holtfreter and reared until the muscular response stage.

Controls were made exactly in the same way except that no membrane was inserted. Eight experimental and six control specimens were studied.

**Series C**

The membranes had A.P.D. 4 mm, and thickness 0.031 mm.; and A.P.D. 4.5 mm, and thickness 0.164 mm. Presumptive ectodermal parts were excised from early gastrulae of *Triturus alpestris* and sandwiches were made with early organizer and a small piece of membrane. The membrane was large enough to shield a part of the ectoderm completely from the organizer. The inner layers of the two pieces of ectoderm faced each other. These sandwiches were reared at room temperature (18°-20° C.) for at least 3 days. During this period the solution was changed once. Only healthy specimens were preserved. In this series twenty-one experimental and twenty-two control specimens were studied.

**RESULTS**

Series A and B showed that the formation of the neural tube can be prevented by the interposition of a membrane between the axial mesoderm and the overlying presumptive neural plate ectoderm. It can be seen in the Plate, fig. 1, that each somite on either side has induced a separate neural tube from the overlying ectoderm in contact with it. These induced tubes are not well differentiated, but the nuclear orientations of the induced tubes (and of the somites) are clear. In another case (Plate, fig. 2) the membrane is a little displaced from its original position, but still separates the mesoderm and ectoderm.

In the control embryos of Series B the axial mesoderm, even as late as the early neural plate stage, induced a neural tube from the grafted presumptive ectoderm (Plate, fig. 3). The induced neural tube is more or less perfect in shape and in differentiation; it is lodged between the somites, in close contact with the notochord. Its development seems to depend mostly upon the chorda and, to a lesser extent, upon the somites.

When a membrane was placed beneath the grafted piece of ectoderm, the part lying over the membrane did not react (Plate, fig. 4). In this figure the neural
tube-like structure induced laterally to the membrane on one side is more advanced in its differentiation and development than that on the other side, and a part of it is still in contact with the chorda as well as somite; the other, in contact only with somite, shows only a neural palisade. The same picture is repeated in another specimen. Here there is again an asymmetry between the two small neural elements induced laterally to the membrane, although the larger element is quite separated from the chorda. Again, the part of the grafted piece of ectoderm lying over the membrane has completely failed to react (Plate, fig. 5).

In series C, the controls had induced neural structures which were better than those of the sandwiches containing membranes. With both the thick and the thin membranes, the induced neural palisade lies only on the side next to the organizer (Plate, fig. 6), and is absent over the membrane.

In all these three series no cytoplasmic connexion across the membrane could be observed.

**DISCUSSION**

The experiments reported above have not revealed any evidence of the passage of inducing influences through micropore membranes, and thus tend to confirm the older impression of the indiffusibility of the natural primary evocator through spaces filled with water or tissue fluids. It may be pointed out, however, that another explanation of the failure of induction is possible. It may be that the quantity of active substance available from the natural organizer during gastrulation is near the effective minimum. It has often been suggested that the area of the neural plate is limited by the diffusion of the active inducing agent from the central region of the archenteron roof towards the sides, the edges of the plate marking the position in which the concentration falls below the threshold of action (cf. Nieuwkoop et al., 1952; Waddington, 1956). If this were the situation, the evocator might still diffuse through the membrane but fail to attain a high enough concentration at the other side to be effective. One could in this way safeguard the hypothesis that the natural evocator of the gastrular archenteron roof is similar in chemical nature to the active substances which have been extracted from various heterologous inductors. On the other hand, it would certainly be most unwise to neglect the possibility that the inducing action of the archenteron roof depends on a cell-to-cell contact, as Weiss (e.g. 1950) has argued is the case for the lens-inducing action of the optic cup. In view of the negative character of the results reported here, this is perhaps not the occasion to attempt a lengthy discussion of the pros and cons of these alternative possibilities.

**SUMMARY**

1. Presumptive neural plate ectoderm of an early gastrula, when separated by a porous membrane (average pore diameters, 4 μm) from the axial mesoderm, failed to differentiate into neural tube either in Axolotl or in Xenopus.
2. In ectodermal sandwich experiments induction always occurred on the side containing the organizer in *Triturus alpestris* and was prevented by the interposition of a porous membrane, when the latter had an average pore diameter of 4 m\(\mu\) or 1.45 m\(\mu\).

3. No cytoplasmic connexion across the membrane could be observed.

4. The lack of induction through the membranes might be due either to a total failure of diffusion of an active substance, or to a failure of a diffusing substance to attain a high enough concentration on the other side of the membrane, or the induction may essentially involve cell-to-cell contact. No decision between these alternatives is reached.

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**REFERENCES**


**EXPLANATION OF PLATE**

**FIG. 1.** Transverse section through the trunk zone of an *Axolotl* embryo. The membrane (which has been dissolved during histological preparation) completely separates the presumptive neural plate from the chorda. On both sides the somites have induced neural tube-like structures. \(\times 160\).

**FIG. 2.** Transverse section through the trunk zone of an *Axolotl* embryo. The membrane is slightly displaced, but still separates the ectoderm and mesoderm. On either side, somites have induced neural tube-like structures. \(\times 130\).

**FIG. 3.** Transverse section through the trunk zone of a *Xenopus laevis* embryo. No membrane. The neural tube is formed from the grafted piece of presumptive early gastrula ectoderm.

**FIG. 4.** Transverse section through the trunk zone of a *X. laevis* embryo. The membrane completely separates the grafted piece of presumptive ectoderm from the chorda. \(\times 115\).
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Fig. 5. Transverse section through the trunk zone of a *Xenopus* embryo. The membrane completely separates the grafted piece of presumptive ectoderm from the chorda. On both sides the induced neural tubes are not well differentiated due to an early fixation. × 60.

Fig. 6. Transverse section of an ectodermal sandwich of *Triturus alpestris*. Part of the ectoderm separated by the membrane is completely non-reactive and the neural palisade is on the side containing the organizer. ×100.

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