The influence of lithium on the competence of the ectoderm in *Ambystoma mexicanum*

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WITH TWO PLATES

INTRODUCTION

The influence of lithium on the amphibian egg has been the subject of a number of investigations. From the work of Lehmann (1937), Töndury (1938), and Pasteels (1945) it is known that exposure of amphibian embryos to lithium results in a progressive cranio-caudal reduction of the central nervous system and a simultaneous conversion of the presumptive notochord into somites. Whereas these experiments were made with whole embryos, attempts have been made in recent years to localize the lithium effect by transplanting or explanting specific parts of the embryo. Gallera (1949), for instance, concluded from his experiments with transplants containing lithium treated presumptive chorda mesoderm, that lithium had reduced the ‘morphogenetic potential’ of this inductor. Lombard (1952), on the other hand, claimed that the susceptibility of amphibian eggs towards lithium was the result of the ion’s direct influence on the ectoderm rather than on the presumptive archenteron roof. Further work by Masui (1959, 1960) showed that if the invaginated prechordal plate of *Triturus pyrrhogaster* gastrulae was treated for 4 hr. with a solution of 0·06 M LiCl, it was no longer able to induce forebrain in competent ectoderm. This repression of prosencephalon was accompanied by the appearance of rhombencephalon. Ogi (1961) showed that a similar shift in neural pattern occurred when competent ectoderm instead of the inductor was pre-treated with lithium.

It was this interesting work of the Japanese authors which attracted our attention some years ago. In the first place we considered it of importance to know whether it would be possible to confirm and extend their observations. For this purpose Masui’s experiments were modified by making use of a different prosencephalic inductor. The anterior notochord of neurulae of stage 15

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(Harrison) was chosen by us because its strong prosencephalic inducing properties had already been analysed by Sala (1955).

In the second place we were interested in certain aspects of the more recent work of Masui (1961) on presumptive ectoderm treated with lithium and cultured without an inductor. Masui had made it clear in his study that lithium itself was capable of inducing mesoderm as well as entoderm. A direct effect of lithium on disaggregated ectodermal cells had already been observed by Barth & Barth (1959, 1962). These authors found that lithium chloride at low concentration or for short exposures converted presumptive epidermis of *Rana pipiens* into nerve cells. Increasing either the exposure time or the concentration led to the transformation of the ectodermal cells into pigment cells, whilst very drastic treatment with LiCl led occasionally to the formation of muscle cells. As neither Masui nor Barth exposed their isolated material to the lithium solution for more than a few hours it seemed of interest to compare the structures induced after a short lithium treatment (2 hr.) with those induced after a longer period of treatment (24 hr.).

The third question which was considered concerned the periods of competence of the ectoderm to various inductive stimuli. In this connection it should be mentioned that the period of neural competence had already been examined by Holtfreter (1938), Gallera (1952), Chuang (1955) and Nieuwkoop (1958).

Holtfreter (1938) aged gastrula ectoderm *in vitro* before grafting it into the dorso-lateral side of host neurulae. He found that the neural competence of the explanted gastrula ectoderm was lost within 12–15 hr.

Gallera (1952) aged presumptive ectoderm from *Triturus alpestris* gastrulae by culturing it in the form of sandwiches in Holtfreter solution. He then transplanted this ectoderm to the exposed anterior region of the archenteron roof of young neurulae, and showed that the reactivity of the ectoderm declined progressively.

Chuang (1955) subjected isolated pieces of ectoderm of *Cynops orientalis* of different developmental stages to a sublethal cytolysis, viz. by disaggregating them in a calcium-free medium followed by reaggregation in a calcium-containing medium. It was known from the work of Holtfreter (1947) that such treatment caused gastrula ectoderm to form prosencephalic neural structures. Chuang found that the ectoderm was already capable of being neuralized 24 hr. before the onset of gastrulation and that the neural competence disappeared by the end of gastrulation.

Nieuwkoop (1958), using his fold implantation technique, showed that for *Ambystoma mexicanum* the period of competence for prosencephalic induction (activation) reached up to stage 11½ (Harrison). Nieuwkoop (1958) and Nieuwkoop & Van der Grinten (1961), suggested further than the period of competence for transformation of the neuralized ectoderm into rhombencephalon and spinal cord extended to later stages, viz. up to stage 13–13½ (Harrison).

In contrast to the rather extensive analysis of neural competence, only a few investigations have been carried out on the mesodermal competence of the
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ectoderm. After the initial studies of Toivonen (1953) on the mesodermalizing action of bone marrow, only Pasteels (1954), Kuusi (1961) and Leikola (1962) have worked on certain aspects of this problem.

Pasteels (1954) transplanted and explanted the ectoderm of centrifuged amphibian embryos at different stages of development. By such treatment he was able to induce mesodermal as well as neural structures in early blastular ectoderm of *Xenopus laevis* and of *Rana temporaria*.

Kuusi (1961) suggested that two inducing factors exist, viz. a mesodermalizing and a neuralizing one acting during separate phases of competence. She emphasized the rôle of the rate of resorption in the induction process.

Finally Leikola (1963) aged ectodermal explants of *Triturus vulgaris in vitro* before implanting a mesodermal inductor. He showed that the mesodermal competence is lost at an earlier stage than the neural competence.

From the observations mentioned above we concluded that it would also be interesting to find an answer to the following question; 'During which period of development, and to what extent, is the presumptive ectoderm sensitive to the mesodermalizing action of the lithium ion'?

MATERIAL AND METHODS

Ectodermal sandwiches enclosing an inductor

In these experiments the presumptive neural and epidermal ectoderm was isolated from early gastrulae (stage 10½ Harrison). The anterior part of the notochord—roughly one-third of its total length—from neurulae of stage 15 was used as inductor. The exact location of the inductor was found by making a longitudinal incision along the mid-line of the neural plate at the level where the two folds begin to approach each other. The neurodermal covering of the notochord was then removed and the inductor was cut free from the attached lateral mesoderm from the point of incision up to the pre-chordal plate. The latter could be distinguished due to the flattening of the archenteron roof at its anterior end. The inductor was then enclosed in two pieces of ectoderm isolated from one gastrula. Control explants were cultured for 2 weeks in undiluted Holtfreter solution containing 20,000 I.U. penicillin G and 0.1 g. streptomycin sulphate per litre. The lithium treated explants were operated and reared during the first 24 hr. in half-strength Holtfreter solution containing 2.1 g. LiCl per litre (1/20 M LiCl) and the same amounts of antibiotic. As in the controls they were transferred to undiluted Holtfreter for the remainder of the 2 weeks.

All explants were fixed in Smith's formol-dichromate for 4 hr., embedded in paraffin, sectioned at 8 μ and stained with Delafield's hematoxylin-eosin.

Ectodermal sandwiches not enclosing an inductor

In this case the ectoderm isolated from embryos of various developmental stages was treated with lithium for 2-hr. or 24-hr. periods. It was found that the
TEXT-FIG. 1. The effect of a 24-hr. lithium treatment on the various neural and placodal structures formed in explants containing anterior notochord as inductor ($n =$ number of explants).
ectoderm of blastulae was difficult to fold into two pieces. Explants from these stages were therefore made by combining the presumptive ectoderm of two embryos. From stage 10 onward sandwiches could be made from the ectoderm of a single embryo.

The explants were reared in a similar manner to those containing an inductor. They had often to be transferred to new culture dishes during the first week because of the shedding of necrotic cells. The operations were carried out in the period March 1961 to June 1962. For some unexplained reasons poor results were obtained from eggs laid from January until April 1962.

RESULTS

**Explants enclosing an inductor**

In Text-fig. 1 a graphical evaluation is given of the neural structures found in thirty-two control explants and in thirty-two explants treated with lithium during 24 hr. The values represent the percentages of explants containing certain structures and are therefore of a qualitative nature only.

From the figure it is evident that lithium treatment has caused a shift in the pattern of neural induction. In our controls the anterior notochord induced, as was to be expected from the work of Sala (1955), predominantly prosencephalon and only small amounts of rhombencephalon. After lithium treatment the situation is reversed: rhombencephalic and spinal cord structures being encountered more frequently than prosencephalic ones.

In Plate 1, Fig. A, a reproduction is given of a part of a typical prosencephalon induced in a control. We find here a telencephalic structure with an olfactory placode and a diencephalic one containing an eye with lens and tapetum. Plate 1, Fig. B, is an example of a section through a lithium treated explant. Here, a well-defined rhombencephalon with cephalic ganglia, an ear vesicle and a spinal cord can be observed.

**PLATE 1**

**Fig. A.** A section through the prosencephalon induced by anterior notochord in a control explant. Parts of the telencephalon (tel.) with olfactory placode (olf.), diencephalon (di.) with eye rudiment (eye) and lens (l.) and atypical epidermis (at. ep.) are shown.

**Fig. B.** A section through the rhombencephalon (rh.) induced by anterior notochord after a 24-hr. lithium treatment of the explant. Besides a large rhombencephalon (rh.) it contains cephalic ganglia (c.g.), an ear vesicle (e.v.) and a spinal cord (sp.c.).

**Fig. C.** An ectodermal explant from a blastula 1 day after a 2-hr. lithium treatment. Contraction of the thickened ento- and mesodermalized edges (e.m.) has resulted in a protrusion of the epidermal half (ep.) of the explant.

**Fig. D.** An ectodermal explant from a blastula 2 days after a 2-hr. lithium treatment. A segregation into a massive ento-mesodermal half (e.m.) and a vesicular epidermal half (ep.) has taken place.
Expiants not enclosing an inductor

In Text-fig. 2 the structures observed after a 24-hr. lithium treatment are given as a function of the stage of development of the ectoderm at the beginning of the experiment. Likewise, in Text-fig. 3 a frequency diagram of the structures formed after a 2-hr. lithium treatment is reproduced. The control series cultured in Holtfreter differentiated only into atypical epidermis.

Perhaps the most striking change observed in the blastula ectoderm after a 2- or a 24-hr. lithium treatment, was the violent cell displacement. It occurred within 48 hr. of the culturing of the explants. This macroscopically visible process was often accompanied by intensive cell expulsion. It can be divided into a number of stages, see Plate 1, Figs. C, D. In Fig. C an intermediate state is shown of a 'hat-shaped' type of explant encountered 24 hr. after lithium treatment. The swollen rim, which represents the original wound surface, can be clearly distinguished from the unaffected central part. The former often segregated from the rest of the explant during the next day (see Plate 1, Fig. D). Explants of the shape shown in Plate 1, Fig. D, were given the name 'pseudo-exogastrulae' because they reminded us strongly of the exogastrulae described by Holtfreter (1933). (Compare, for instance, our Plate 1, Fig. D, with Holtfreter's Fig. 30, page 737, and Fig. 36, page 756.)

After 2 weeks of culture an histological analysis of both parts of the explants could be made. In Plate 2, Fig. E, large epidermal vesicles formed in the upper half of the explant (part ep. of Fig. D) are shown. These vesicles often contained some necrotic elements. In Plate 2, Fig. F, a section through the lower massive and often spherical half of the explant (part e.m. of Fig. D), is given. In general, this part differentiated rather poorly and contained mainly yolk-laden cells. Occasionally, however, myofibrils arranged in somites and pronephric tubules were formed. Of especial interest to us was the outer layer of highly cylindrical, vacuolated cells, to be seen in Plate 2, Fig. F. These cells were always underlain

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**PLATE 2**

**Fig. E.** The 'epidermal' part of a lithium treated explant from early gastrula ectoderm. Inside the epidermal vesicles some necrotic elements (n.e.) are present.

**Figs. F, G and H** are sections through the ento- and mesodermal part of lithium treated explants of blastula or early gastrula ectoderm.

**Fig. F.** The mesoderm is arranged in somites (som.). The outer entodermal epithelium (ent.) is underlain by a thin layer of smooth muscle cells (sm.m.).

**Fig. G.** The muscular elements have formed somites. Nephrogenous tubules (neph.) and yolk-rich undifferentiated elements (y.e.) are present. On one side the covering layer of the explant is epidermal (ep.) with a few subcutaneous chromatophores (chr.) whilst on the other side an entodermal layer (ent.) with underlying smooth muscle cells is found (proct. = proctodeum).

**Fig. H** shows a more complex organization of mesodermal structures. It consists of notochord (not.) surrounded by somites (som.), cardiac? muscle cells (car. m.), pronephric tubules (neph.), some necrotic blood elements (bl.) and yolk rich undifferentiated elements (y.e.).
PLATE 2

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(Facing page 322)
TEXT-FIG. 2. The mesodermal and endodermal structures formed after a 24-hr. lithium treatment of ectodermal explants not containing an inductor. The different stages of development of the ectoderm, when lithium treatment was begun, are indicated by different types of shading of the columns. Untreated controls (not included) differentiated into atypical epidermis only ($n =$ number of explants).
TEXT-FIG. 3. The effect of a 2-hr. lithium treatment on ectodermal explants not containing an inductor. For conventions see legend to Text-fig. 2.
by a sheet of smooth muscle cells. In contradistinction with an epidermal covering, which usually consists of two cell layers and which is often underlain by chromatophores, this epithelium was one layer thick and never underlain by chromatophores. It closely resembled the entodermal covering layer of Holtfreter's exogastrulae and was identified as an endodermal type of epithelium.

In Plate 2, Figs. G, H, sections are shown of explants containing a variety of structures. In Fig. G somite formation of muscle cells (som.) has taken place. Fin, proctodeum and pronephric tubules have also developed. Masses of yolk-laden undifferentiated elements (y.e.) are often observed. The right half of this section has a covering of cylindrical entodermal cells (ent.) which are underlain by a layer of smooth muscle cells. In the upper left of this section the covering is epidermal (two layers thick) with a number of subcutaneous chromatophores. Finally in Fig. H of Plate 2, a section through an ectodermal explant taken from a blastula and treated for 2 hr. with lithium, is shown. After such a short treatment with Li the cellular differentiation of the explants was better than after a 24-hr. exposure to the Li solution. In Fig. H a notochord (not.) can be seen which is surrounded by muscle cells arranged into somites. Other structures identified include blood cells, yolk elements, pronephric tubules and probably cardiac muscle cells. In a number of explants the notochord was accompanied by two rhombencephalic neural tubes. The significance of this finding is considered in the discussion on page 327.

**DISCUSSION**

From Text-fig. 1 we learn that a shift in neural induction pattern occurs when an ectodermal explant containing a prosencephalic inductor is treated with lithium. This is therefore a confirmation of Masui's original observation on the effect of lithium on the inducing capacity of gastrula pre-chordal plate. Masui's experimental procedure, however, differed somewhat from our own. In our case, ectoderm and inductor were immediately combined after the operation in the Li solution. Such a procedure has the advantage that the uncoated inner surface of the ectoderm and also the inductor are not exposed to the lithium solution for more than a few minutes. Wound closure can therefore take place rapidly. On the other hand, our experiments cannot give an answer to the question whether lithium acts preferentially on the ectoderm or on the inductor. According to Masui (1959) lithium treatment either of the ectoderm or of the inductor will give a similar shift in the neural induction pattern from prosencephalon to rhombencephalon.

These results, based, unfortunately, on a qualitative analysis only, nevertheless allow us to speculate on the mode of action of the lithium ion. In our opinion it seems likely that the lithium effect is related to the process of activation by phenol (see Becker et al., 1961). These authors found that a prosencephalic inductor isolated from chick embryos could be turned into a rhombencephalic inductor on phenol extraction. As, according to Saxén & Toivonen (1962), a hindbrain
Inductor is always composed of a mesodermal and a neural (prosencephalic) inductive component, Becker et al. concluded that phenol had activated a mesodermal inductive factor. The activated mesodermal factor was also found to suppress the neural, prosencephalic one because on (re)-inactivation of the mesodermal factor by heat Becker et al. could demonstrate the prosencephalic factor again. On the basis of these results we would like to suggest that lithium activates, in a manner similar to phenol, a mesodermal inductor or represses a mesodermal inhibitor.

It is, however, unlikely that lithium should act only in this way on the explant. In fact the complex nature of its action follows from other properties such as its toxicity as judged by the number of necrotic cells and its inhibitory effect on notochord formation after a 24-hr. treatment.

If we now turn our attention to the effect of lithium on ectoderm not enclosing an inductor, we are immediately struck by the conformity between the graphs of Text-fig. 2 and Text-fig. 3. With the exception of notochord formation, it does not appear to make any difference how long the explants are brought into contact with lithium. Nevertheless we are under the impression that a shorter treatment reduces the number of necrotic cells and leads to a quantitative increase in the total amount of differentiated induced structures.

Of special interest is the high incidence of muscle and pronephros induction in blastula ectoderm. Masui (1961) and Barth & Barth (1962) found these structures only in a few cases in their experiments. However, these authors worked with gastrula ectoderm which, according to our Text-fig. 2 and Text-fig. 3, is no longer the optimal stage for mesodermalization. It also follows from these figures that the period of mesodermal competence ends with stage 11 (Harrison). Recently, Tseng Mi-Pai (1963), using bone marrow as inductor, found a similar disappearance of the mesodermal competence in Cynops orientalis at the horse-shoe shaped blastopore stage. Ageing of the ectoderm, moreover, resulted in a dorso-ventral shift of the type of mesodermal differentiations.

Ectoderm younger than stage 8 was not cultured owing to the technical difficulty of making explants from thick pieces. We were therefore unable to determine the exact stage when mesodermal competence is initiated in the ectoderm.

Concerning the problem of morphogenetic movement of Li treated blastula ectoderm, it has already been mentioned that a relation was believed to exist between this process and the movements taking place during exogastrulation (Holtfreter 1933). If we are correct in the interpretation that the palisade structure in our explants is an entodermal epithelium, we have another argument in favour of some sort of kinship between our segregated explants and Holtfreter's exogastrulae. As the segregation process begins within a number of hours after lithium treatment the transformation of ectoderm into entoderm must be a rapid process. The lack of morphogenetic movement of lithium treated ectoderm from older gastrulae may also be taken as a proof that the meso-entodermal competence phase does not extend further than stage 11 (Harrison).
Another question deserving discussion is why explants containing anterior notochord as inductor do not form any significant amounts of mesodermal or entodermal structures. A possible explanation may be the following: the sandwiches were made with ectoderm of stage 10.5 (Harrison). This stage is no longer optimal for mesodermalization (see Fig. 2), but its competence for neuralization is still undiminished (Chuang, 1955; Nieuwkoop, 1958). Through a possible interaction of the two competences only small amounts of mesoderm may be induced whilst the amount of neural structures is high.

It has also to be explained why ectoderm of stage 8 and 9 (Harrison), after a short lithium treatment, not only forms notochord but sometimes also rhombencephalic neural tubes. Most likely the neural tubes are not directly formed by the action of the lithium. They are probably induced indirectly by the notochord in adjoining ectoderm in those explants where incomplete segregation of entomesodermal and epidermal components has taken place. As the neural competence of blastula ectoderm in vitro is lost within 24 hr., this implies that histologically undifferentiated cells, destined to become notochord, must induce neural structures shortly after being determined themselves by lithium.

Finally we would like to consider the different periods of competence of the presumptive ectoderm for various inductive actions.

Nieuwkoop (1958) and Nieuwkoop & van der Grinten (1961) concluded that besides the mesodermal competence there were two separate neural competences, viz. a competence for activation (neuralization) of the still 'virgin' ectoderm and a competence for transformation (caudalization) of the activated ectoderm. The first competence for prosencephalic neural differentiation extends in Ambystoma up to stage 11½; the second for mes- and rhombencephalic and spinal cord differentiation extends probably slightly beyond stage 13 (Harrison) (see Text-fig. 4).

Leikola (1963) observed in his studies with aged ectoderm of Triturus vulgaris that the neural competence phase extends to later stages than the mesodermal one. During the stages in which the ectoderm was still competent for both influences, the neural differentiations were of a rhombencephalic or spino-cordal character. After the disappearance of the mesodermal competence the induced neural structures were only of a prosencephalic nature, notwithstanding the presence of the mesodermalizing principle. This observation is a strong argument against a possible identity of the mesodermalizing and transforming principle. In Leikola's experiments, as well as in those of many other authors, transformed neural structures are only formed in the presence of mesodermal differentiations.

Takata & Yamada (1960) have already demonstrated that the inductive action of bone marrow led to the formation of mesodermal and also of entodermal structures. We, too, have found that blastula ectoderm treated with lithium will form both mesodermal as well as entodermal structures. However, our experiments do not support Tseng Mi-Pai's (1963) observations. She found no decrease in the amount of entodermal differentiations in ectoderm of Cynops orientalis of
which the mesodermal competence was reduced. We observed that for *Ambystoma mexicanum* the mesodermal as well as the entodermal differentiations decreased with increasing age of the ectoderm. It is evident that more work will have to be done before an answer can be given to the question whether separate competence phases also exist for entodermalization and mesodermalization of the ectoderm.

Text-Fig. 4. The successive phases of competence for (1) ento- and mesodermal, (2) prosencephalic neural, and (3) rhombencephalic and spino-cordial neural differentiation of the ectoderm are presented. In the absence of any inductive action, the ectoderm develops exclusively in an epidermal direction. On the abscissa stages are given according to Harrison.

**SUMMARY**

1. Ectodermal sandwiches of *Ambystoma mexicanum* of stage 10\(\frac{1}{2}\) (Harrison) containing anterior notochord as inductor were treated with a 0·05 M LiCl solution for 24 hr. It was found that lithium was capable of changing the neural induction pattern of these explants by increasing the number of rhombencephalic and spino-cordial inductions and decreasing the number of prosencephalic ones.

2. Sandwiches made from ectoderm of various developmental stages (stages 8–12) and not containing an inductor were treated with the lithium solution for 2- or 24-hr. periods. In the explants made with ectoderm of stage 8, stage 9 or stage 10\(\frac{1}{2}\) a very characteristic type of cell displacement occurred which resembled the phenomenon of exogastrulation. The ectoderm of the younger stages was
shown to be mesodermalized and entodermalized to a considerable extent by the treatment.

3. The period of mesodermal competence of the ectoderm, as judged by its reactivity towards lithium, extends from stage 8 (or earlier) up to stage 10 1/2/11 (Harrison).

4. The different periods of primary competence for (a) meso- and entodermalization, and (b) activation (neuralization) of the ectoderm are considered. Also the subsequently acquired competence for (c) transformation (caudalization) of the activated ectoderm into more caudal neural structures is discussed. The various periods of competence are presented in the form of a diagram together with the corresponding inductive stimuli.

**RÉSUMÉ**

*Influence du lithium sur la compétence de l'ectoderme chez* 
*Ambystoma mexicanum*

1. Des sandwichs d'ectoderme d'*Ambystoma mexicanum* au stade 10 1/4 (Harrison), contenant de la notochorde antérieure comme inducteur, ont été traités par une solution de LiCl 0,05 M pendant 24 heures. On a trouvé que le lithium est capable de modifier l'allure de l'induction neurale de ces explants en accroissant le nombre d'inductions rhombencéphaliques et spino-caudales et en diminuant celui des inductions prosencéphaliques.

2. Des sandwichs formés d'ectoderme pris à divers stades du développement (stade 8–stade 12) et ne contenant pas d'inducteur ont été traités avec la solution lithinée pendant des durées de 2 ou 24 heures. Dans les explants faits avec de l'ectoderme du stade 8, du stade 9 ou du stade 10 1/4, est survenu un déplacement cellulaire très caractéristique qui ressemblait au phénomène d'exogastrulation. On a montré que l'ectoderme des stades plus jeunes est considérablement 'mesodermalisé' et 'endodermalisé' par le traitement.

3. La période de compétence mésodermique, si on en juge par sa réactivité envers le lithium, s'étend du stade 8 (ou avant) jusqu'au stade 10 1/2/11 (Harrison).

4. On envisage les différentes périodes de compétence primaire pour (a) la méso- et l'endodermalisation, et (b) l'activation (neuralisation) de l'ectoderme. On discute aussi la compétence acquise par la suite pour (c) la transformation (caudalisation) de l'ectoderme activé en structures neurales plus caudales. Les différentes périodes de compétence sont présentées sous forme d'un diagramme avec les stimuli inducteurs correspondants.

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