

## Lithium inhibits morphogenesis of the nervous system but not neuronal differentiation in *Xenopus laevis*

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### Summary

*Xenopus* embryos treated with 100 mM-lithium from the 2- to 4-cell stage to the early blastula stage (4 h) failed to neurulate and developed without a discernible anteroposterior axis. The internal structure of defective embryos was grossly disorganized, but immunohistochemical staining with cell-type-specific antibodies revealed differentiated nerve and muscle cells. Quantitative assay in tissue cultures from control and acutely abnormal lithium-treated embryos showed that neural differentiation was enhanced and muscle differentiation unaffected. The embryos took up about 0.5 mM-lithium at threshold, maximal effects resulted at 2–3 mM. Most of the lithium was extruded from the cells into the blastocoel fluid, where lithium reached 17 mM. The threshold intracellular concentration was about 150  $\mu$ M. Lithium uptake rose steeply as the osmotic/ionic strength of the bathing medium

increased. Sodium, potassium and lithium were equally able to increase the permeability of the embryo. However, sodium ions enhanced, while potassium ions interfered with, the uptake of lithium. Treatment with lithium at progressively later stages reduced the developmental defects and neural differentiation returned to normal levels. The uptake of lithium did not decline concomitantly. We conclude that lithium does not inhibit neural induction, but interferes with dorsal patterning. The sensitivity of the embryo to lithium is determined by developmental stage. The very low, effective intracellular concentrations may be important in understanding the mechanism of lithium-generated defects.

Key words: lithium, *Xenopus laevis*, neural development, neurulation.

### Introduction

The teratogenic consequences of exposure of early sea urchin and amphibian embryos to lithium ions have been recognized for many years. In the sea urchin, lithium treatment of the early embryo leads to the over production of structures derived from the vegetal pole, at the expense of structures derived from the animal pole (Herbst, 1892). In the amphibian embryo, lithium treatment affects the development of the notochord and causes a reduction in neural structures such as the optic rudiments (Morgan, 1903). Backstrom (1954) obtained similar results and further suggested that neural differentiation was reduced. Consequently he proposed that lithium 'ventralized' the embryo.

Both sea urchin and amphibian embryos are most sensitive to lithium treatment during the early cleavage stages (sea urchin: Lallier, 1964; amphibian:

Pasteels, 1945; Hall, 1942; Backstrom, 1954). By gastrulation, exposure of the embryos to lithium generates less-marked defects and once gastrulation is complete lithium treatment has little effect. Attempts to clarify the consequences of lithium treatment by exposing isolated ectoderm stripped from the amphibian embryo to lithium have demonstrated a confusingly wide range of effects. These include the induction of derivatives characteristic of all three germ layers (Englander & Johnen, 1967), the formation of mesodermal structures (Ogi, 1961; Masui, 1961; Grunz, 1968; Johnen, 1970) and the induction of neural differentiation (Barth & Barth, 1962, 1974).

In this paper we examine the consequences of treatment of early amphibian embryos with lithium ions in order to test whether previous suggestions that the lithium ion might interfere with neural induction are correct. We have used the appearance of differentiated neurones as one criterion of successful neural

induction. Phenotypically differentiated neurones are identified in intact embryos using cell-type-specific antibodies and quantitative assessment of the extent of neural differentiation is made using an *in vitro* assay (Messenger & Warner, 1979; Breckenridge & Warner, 1982). We conclude that neural differentiation is not prevented by lithium treatment, despite the gross disturbance of morphogenesis and patterning. This conclusion parallels that drawn in a very recent paper by Kao, Masui & Elinson (1986), who examined pattern formation in *Xenopus laevis* after treatment of cleavage-stage embryos with lithium. These authors suggest that lithium enhances the development of dorsoanterior structures and that this respecification of the pattern-forming system is responsible for the morphological appearance of the treated embryos.

The mechanism by which lithium ions generate these effects on development is not known. Surprisingly, there are no estimates of the intracellular concentration of lithium that is necessary to generate the developmental defects. We have therefore measured the concentration of lithium within amphibian embryos after treatment, for comparison with the teratogenic effects.

Some of these results were included in a thesis submitted to the University of London in part fulfilment of the requirements for the Ph.D (Breckenridge, 1985).

## Materials and methods

*Xenopus laevis* embryos were obtained from adult frogs induced to mate and lay by injection of chorionic gonadotrophin (Pregnyl, Organon Ltd). Embryos were reared in tap water until they reached the required developmental stage; staging follows the normal table of *Xenopus laevis* (Nieuwkoop & Faber, 1956).

### Treatment with lithium and other salts

For each batch of eggs at least 10, more usually 20, embryos of the required stage were soaked in a 100 mM solution of the appropriate salt for the required length of time. When some embryos were to be removed for ion analysis (see below) the number of embryos was increased. The jelly coat was deliberately left intact, even though it could provide a variable barrier to the uptake of lithium, because chemical removal of the jelly coat with cysteine can disturb the distribution of small ions within the embryo (A. Stotter, R. L. Warren & A. E. Warner, unpublished results). An equivalent number of sibling embryos were placed into tap water. At the end of exposure to salt solution, the experimental embryos were washed, returned to tap water and left for 20–24 h, by which time the control embryos had reached stage 20–22. All experiments were carried out at room temperature (18–22°C).

### Assessment of abnormalities

The embryos were assessed for salt-induced abnormalities and batch viability when the control embryos had reached stage 20–22. For each group the number of dead and/or abnormal embryos was determined. The number of embryos that died in the control group gave the spontaneous death within the batch. The percentage of embryos that died spontaneously was subtracted from the percentage that died after each experimental regime, to give the death rate for each condition tested. In individual experiments the effects of the different treatments were compared on sibling embryos from the same batch. Salt-treated embryos were scored for abnormalities as described in the Results section.

### Histology

Control embryos at stage 25–28 and lithium-treated siblings of an equivalent age were fixed overnight in Bouin's fluid, washed in buffer, dehydrated in alcohols and embedded in butoxyethanol-glycol methacrylate (Dupont Ltd). 7 µm serial sections were cut on a Dupont-Sorval JB4 microtome and stained with haematoxylin and eosin.

### Immunohistochemistry

Unfixed embryos at stage 25–28 were embedded in Tissue-Tek, frozen in isopentane and 5–7 µm sections cut on a Bright 5030 cryostat. Sections were incubated for 1 h at room temperature with: (i) a monoclonal antibody raised against rat neurofilament protein (Wood & Anderton, 1981); (ii) a monoclonal antibody (12/101) raised against adult newt muscle (Kintner & Brockes, 1984). The sections were then washed three times with Ringer solution and incubated with FITC-labelled goat anti-mouse antibodies (Nordic Immunological Laboratories, Maidenhead). After further washing to remove unbound antibodies, the sections were mounted in 'anti-fade' mountant (Chemistry Department, City University, London) and viewed on a Zeiss epifluorescence microscope equipped with appropriate filters. Photomicrographs were taken on Kodak Tri-X Pan film.

### Tissue culture

Neural tube, notochord, somitic mesoderm and some overlying ectoderm were dissected from embryos at stage 19–20, when the neural tube had just closed. The dorsal surface and immediately underlying mesoderm were dissected from embryos of the same age, which had previously been treated with lithium. The dissected portions were soaked for 3–5 min in calcium-free Ringer solution (120 mM-NaCl, 2.5 mM-KCl, 2 mM-Tris-hydroxymethyl-aminomethane, 1 mM-EGTA) at pH 8.0 and then dissociated into single cells by mechanical trituration. Material from three embryos was dispensed into each 35 mm culture dish (Falcon Ltd) and cultured at 22°C in modified Ringer solution (100 mM-NaCl, 2.5 mM-KCl, 2.0 mM-CaCl<sub>2</sub>, 2.0 mM-MgCl<sub>2</sub>, 5.0 mM-NaHCO<sub>3</sub>) with 10% fetal calf serum (Gibco-Biocult) and 300 i.u. penicillin/streptomycin (Gibco) at pH 7.4. Three culture dishes were set up from both control and lithium-treated embryos. The cultures were scored 18–24 h after plating, when a monolayer of 60 000 to 70 000 differentiated cells had formed. Neurones,

muscle cells and the total number of cells were counted in 20–30 microscope fields selected at random from the three Petri dishes. Further details of the culture method can be found in Messenger & Warner (1979).

#### *Ion analysis*

At the end of the treatment period, when the embryos had reached stage 7, four to six embryos were removed, washed thoroughly in three changes of double-distilled deionized water and all the jelly removed with clean, flamed forceps. The embryos were divided between two Eppendorf tubes each containing 1 ml of double-distilled, deionized water and broken up by repeated freezing and thawing. The lithium concentration was measured in 1  $\mu$ l aliquots of each sample and the final wash solution by flame emission spectroscopy (Warren, 1980). For calculation of lithium concentration, the volume of the embryo at stage 7 was taken as 1.33  $\mu$ l (Slack, Warner & Warren, 1973). Since the diameter of *Xenopus laevis* embryos can vary from batch to batch, this assumption introduces some error. The range of volumes within all the measurements of Slack *et al.* (1973) was 1.02–1.5  $\mu$ l.

#### *Microinjection*

The jelly coat was mechanically stripped from 2-cell embryos and approximately 1 nl of 1 M-LiCl or 1 M-NaCl injected into each cell, giving an intracellular lithium concentration of about 2 mM. Injection pipettes were pulled from Corning 7740 omega dot glass capillary, backfilled with the injection solution and connected to a Picospritzer (General Valve Corporation). The tip of the pipette was broken back to approx. 5  $\mu$ m. Each injection pipette was calibrated by measuring the volume of a drop expelled from the tip for a given pulse pressure (138–276 kN m<sup>-2</sup>) and width.

#### *Samples of blastocoel fluid*

Micropipettes were pulled from 1 mm external diameter, 0.33 mm internal diameter glass capillary and the tip broken back to 2–5  $\mu$ m. The volume of the pipette tip was measured up to the shoulder. The pipette was inserted between the cells of the animal pole into the blastocoel cavity of embryos at stage 7–8. Blastocoel fluid was rapidly drawn into the tip by capillarity. The pipette was removed from the embryo and placed in a clean tube containing double-distilled, deionized water. Each sample was analysed for potassium and lithium by flame emission spectroscopy (Warren, 1980). Cell damage during insertion or withdrawal of the pipette produced yolky samples and potassium levels well above that normally found in blastocoel fluid (1–4 mM: Slack *et al.* 1973; Gillespie, 1983); contaminated samples were discarded. As observed previously (Slack *et al.* 1973) cytoplasmic contamination was rare.

## Results

### *Observations on the abnormalities generated by lithium treatment*

The majority of embryos treated with 100 mM-lithium from the 2- to 4-cell stage to the early blastula stage (4 h) developed normally until neurulation began. In a few, very severe cases the embryo exogastulated; this was followed by cytolysis and death. Fig. 1 shows a normal tadpole at stage 26 that had been exposed to 100 mM-NaCl (A) and examples of the range of abnormalities seen in lithium-treated embryos (B–F). The defects ranged in severity from slight inhibition of elongation (B), severe inhibition of elongation with an apparent enlargement of the belly (C), complete absence of elongation, with the neural folds failing to close (D) to complete absence of neural folds, which was sometimes accompanied by a long proboscis (E). The most abnormal embryos often died shortly after the controls had reached stage 20–22. A similar range of defects was observed by Backstrom (1954), using the same treatment schedule. Kao *et al.* (1986) treated embryos with higher concentrations of lithium (0.2 and 0.3 M in 20% Steinberg's solution) for a few minutes only and noted equivalent defects.

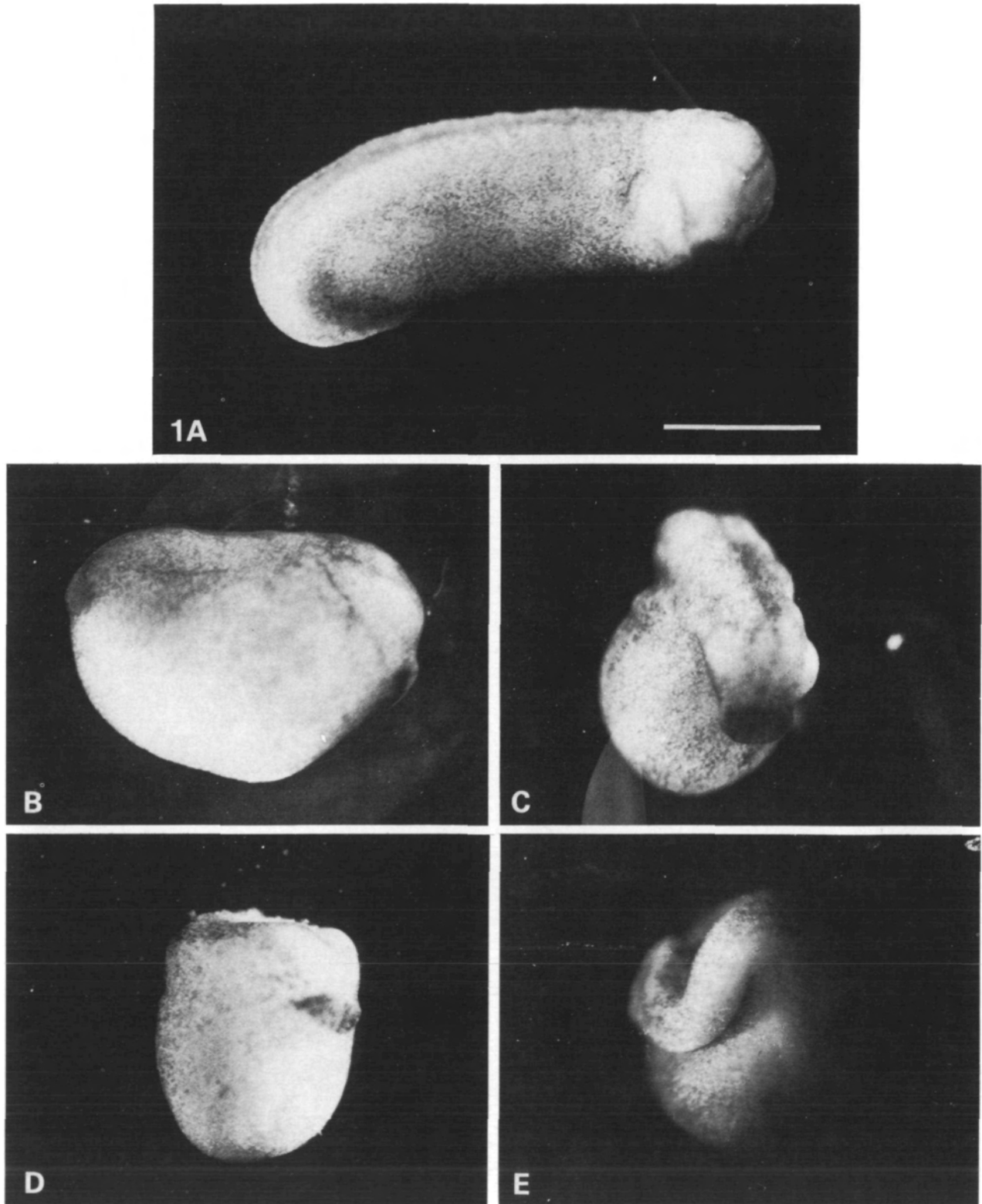
To determine the teratogenic effect of lithium under different experimental conditions, the embryos were scored for the percentage that died within each batch and the percentage showing any of the abnormal features characteristic of lithium treatment, illustrated in Fig. 1. The relative severity of the abnormalities was not determined systematically, but when the percentage of embryos showing abnormalities was low, the abnormalities were less severe. Conversely, a high proportion of dead embryos was accompanied by more severely abnormal embryos. A proportion of the untreated embryos in each batch died also, reflecting differing degrees of viability. All experiments included in this paper were made on batches of high viability (greater than 80%, average 90%). The consequences of lithium treatment showed considerable variability from batch to batch, as seen by Stanistreet (1974). However, occasional batches showing extremely poor viability (20–40%) were no more sensitive to lithium than the rest. The teratogenic consequence of lithium treatment was defined as the sum of dead and abnormal embryos, after correction for the viability of each batch.

### *The uptake of lithium*

In order to resist the net loss of ions during development the membranes of external facing cells of the amphibian embryo are relatively impermeable to ions and water (Slack & Warner, 1973; Slack *et al.* 1973). It is, therefore, difficult to predict how much lithium

enters the embryo even from high-salt solution. Measurements of lithium concentration made at the end of a 4 h exposure to 100 mM-LiCl in embryos

taken from 25 batches gave a mean of  $2.54 \pm 0.18$  mM-lithium (mean  $\pm 1$  standard error; 47 samples each containing two or three embryos), calculated using an



**Fig. 1.** The external appearance of abnormal embryos generated by lithium treatment from the 2- to 4-cell stage to stage 7 (4 h). (A) Normal embryo at stage 26 previously treated with NaCl. (B-F) The range of abnormalities seen in embryos of the same age that had been previously treated with LiCl. For detailed description see text.

embryo volume of  $1.33 \mu\text{l}$  (see Methods section). The large standard error reflects considerable variability in lithium content from embryo to embryo (range:  $0.5\text{--}5 \text{ mM}$ ); part of this variability undoubtedly arises because of differences in embryo volume. Thus very little lithium enters the embryos, despite the high external concentration. Equivalent variability was noted in the teratogenic consequences of lithium treatment of embryos from these batches (abnormal:  $54.9 \pm 7\%$ ; dead:  $32.3 \pm 7.5\%$ ; range (abnormal + dead)  $5\text{--}100\%$ , 349 embryos; spontaneous death:  $13.5 \pm 3.5\%$ ).

Fig. 2A plots the relationship between extra-embryonic lithium and teratogenic effect (solid line), along with the concentration of lithium within some embryos of each batch (dotted line) for four batches of eggs. The developmental effect of lithium is scored as the sum of the abnormal and dead embryos from each batch (60–65 embryos per point), after taking account of spontaneous death in untreated controls ( $8 \pm 2\%$ ; four batches, 60 embryos). An external lithium concentration up to  $50 \text{ mM}$  had no significant teratogenic effect and the embryo lithium concentration was less than  $0.5 \text{ mM}$  (average of  $20\text{--}24$  embryos).  $100 \text{ mM}$ -lithium generated the usual substantial teratogenic effect with an average lithium uptake of  $2.5 \text{ mM}$ , close to the overall average given above.

Lithium uptake and teratogenic effect were similarly related when the time of exposure to  $100 \text{ mM}$ -lithium was varied (Fig. 2B). The spontaneous death in the batches used for these experiments was again close to  $10\%$ . A significant teratogenic effect was seen after 1 h in  $100 \text{ mM}$ -LiCl (seven batches, 100 embryos) by which time the embryos contained, on average,  $0.5 \text{ mM}$ -lithium (eight batches, 16 samples each containing two or three embryos). Both the teratogenic effect and the lithium uptake increased linearly with time of exposure, suggesting a high correlation between the intraembryonic concentration of lithium and the developmental defects. The proportion of severely affected embryos and the proportion that died as a result of lithium treatment increased together. Fig. 2C combines the results given in A and B with data obtained in a number of other experiments (see later) and plots the concentration of lithium within the embryos against the percentage lithium effect, taken as the sum of the abnormal and dead embryos for each batch, after correction for spontaneous death (average  $12\%$ ). The figures at the foot of each column give the number of batches falling into each class, so that there were 30 batches showing a lithium effect of  $0\text{--}10\%$  (440 embryos) with an average lithium concentration of  $0.72 \pm 0.06 \text{ mM}$  (140–180 embryos). The teratogenic effect of lithium is highly correlated with intraembryonic lithium concentration; the crosses

describe a linear regression curve fitted to the data with a correlation coefficient of  $0.82$  ( $P < 0.01$ , *t*-test). The results suggest that the threshold concentration of lithium required to generate developmental abnormalities is close to  $0.5 \text{ mM}$ - and  $2.5 \text{ mM}$ -lithium is sufficient to produce a maximal effect. Similar defects were produced by the microinjection of lithium, into both cells of embryos at the 2-cell stage, to give a final intracellular lithium concentration of about  $2 \text{ mM}$ . Injection of sodium had no effect.

#### *The concentration of lithium in the blastocoel fluid*

To determine the way in which lithium taken up by the embryo is distributed between the cells and the intercellular fluid, samples of intercellular fluid were withdrawn from the blastocoel cavity of embryos that had been soaked in  $100 \text{ mM}$ -LiCl for 4 h. Seven samples that were uncontaminated by cell damage gave a lithium concentration of  $17 \pm 6.3 \text{ mM}$ . In all cases the concentration of lithium in the blastocoel fluid was substantially greater than the average concentration within the whole embryo after 4 h in  $100 \text{ mM}$ -LiCl (average:  $2.5 \text{ mM}$ ). This suggests that a substantial proportion of the lithium taken up by the embryo is extruded from the cells into the intercellular fluid. A rough estimate of the intracellular lithium concentration can be made using the values for blastocoel volume and protein content of the stage-7 embryo given in Slack *et al.* (1973). This calculation suggests that the intracellular concentration of lithium that produces the maximum teratogenic effect may be as low as  $0.8 \text{ mmoles l}^{-1}$  cell water.

#### *The sensitivity to lithium depends on developmental stage*

Fig. 3A shows the developmental consequences of exposure of *Xenopus* embryos to  $100 \text{ mM}$ -lithium for 4 h, initiated at progressively later times during development. At least three time points were tested on every batch. The spontaneous death within the batches used for this series of experiments was  $19\%$ . Each column gives the sum of the abnormal and dead embryos; the hatched portion indicates the proportion that died as a result of treatment, taking into account the batch viability. A 4 h exposure to lithium became progressively less effective as the embryos proceeded through development. Lithium treatment beginning at the 32- to 64-cell stage was significantly less teratogenic than treatment starting at the 2- to 4-cell stage (2- to 4-cell *versus* 32- to 64-cell  $P < 0.02$ , Student's *t*-test) and by stage  $10\frac{1}{2}$ , three batches (48 embryos) were completely unaffected. However, the uptake of lithium did not decline equivalently. Embryos at the 64- to 128-cell stage contained  $1.85 \text{ mM}$ -lithium after 4 h exposure, not significantly different from embryos exposed to LiCl from the 2- to 4-cell

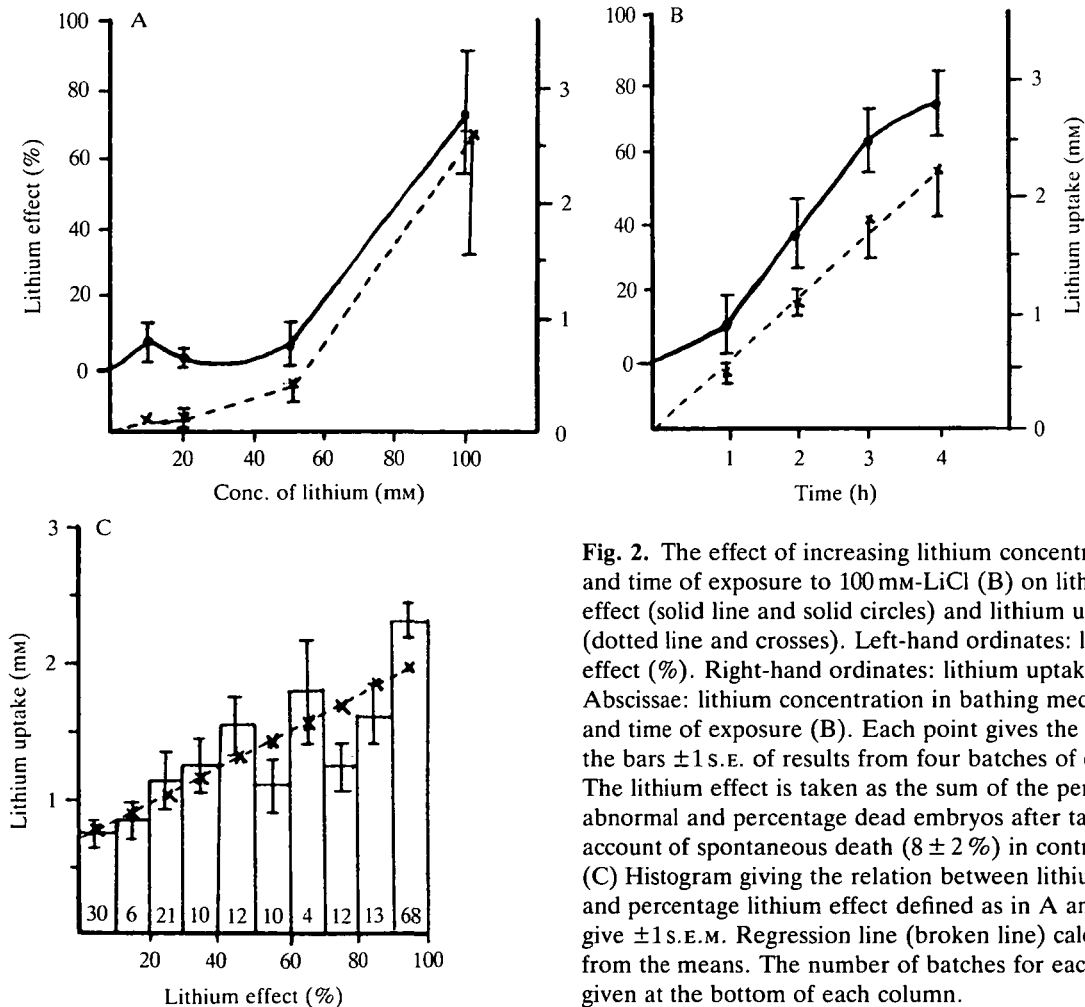
stage onwards. At stage 10½, when lithium treatment no longer produced any developmental defects, the embryos nevertheless contained 1.5 mM-lithium at the end of the 4 h exposure. Since this concentration would have been sufficient to generate a 60 % lithium effect in embryos treated from the 2- to 4-cell stage, *Xenopus* embryos must become less susceptible to the teratogenic effects of lithium with increasing age.

*Internal structure of lithium-treated embryos*

The most characteristic external feature of embryos that have been treated with lithium is the inhibition of the elevation of the neural folds. The internal organization of structures within such embryos also proved to be grossly disorganized. Fig. 4 shows the external appearance of a normal embryo (A) at stage 26 and the dorsal view of two sibling embryos of the same age that had previously been treated with lithium (B,C). Transverse sections through the head (A1, B1, C1) and trunk (A2, B2, C2) of these embryos are shown below. By comparison with the control, both lithium-treated embryos showed gross disorganization of normal patterning. No recognizable organized structures could be found in embryo B, which

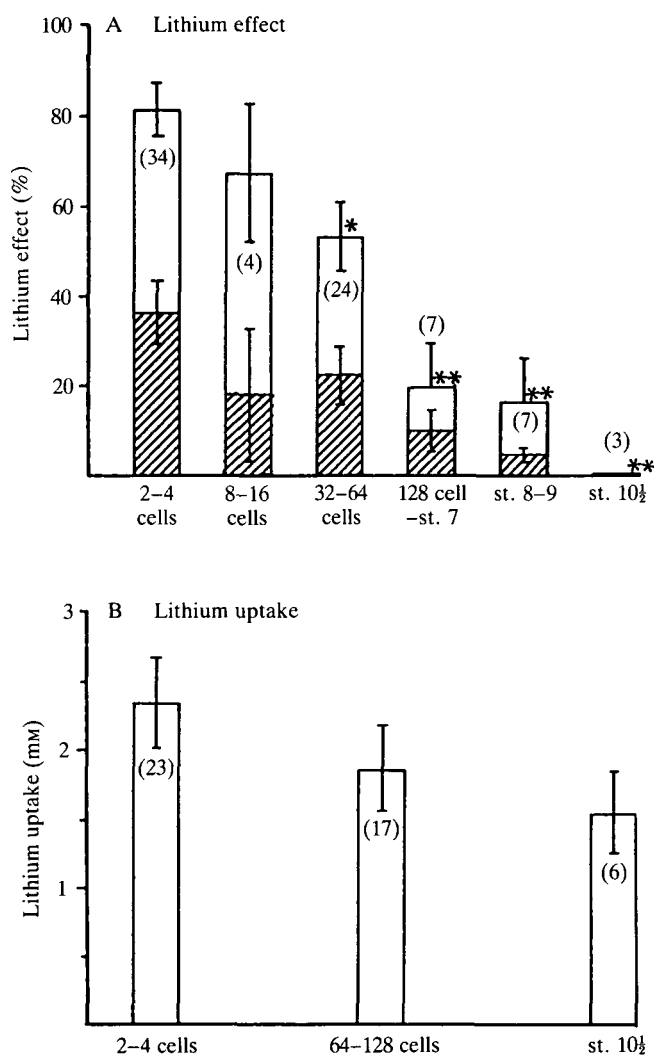
completely lacked the notochord. Embryo C had no recognizable anterior-posterior axis and the internal structures were again disorganized. Patches of apparently differentiated, but unidentifiable tissue, are scattered throughout.

However, when tissue-specific antibodies were applied to frozen sections of such disorganized embryos both differentiated nerve cells and muscle cells could be recognized. Fig. 5 shows horizontal sections through the head of a control embryo (A) at stage 28 and the dorsal region of an abnormal sibling of the same age that had been treated with lithium (C), each labelled with an anti-neurofilament monoclonal antibody (Wood & Anderton, 1971). In the control embryo, neurites emerging from the developing brain, and particularly the otic nerve, are clearly labelled. The pattern of staining in the lithium-treated embryo was quite different, with labelled cells scattered throughout the internal structures. In both control and lithium-treated embryos the antibody also stained epidermal cells. However, incubation of similar sections with an antibody that is specific for epidermal cells (2f7.c7: Jones, 1985) showed staining of the epithelium only, confirming that the scattered



**Fig. 2.** The effect of increasing lithium concentration (A) and time of exposure to 100 mM-LiCl (B) on lithium effect (solid line and solid circles) and lithium uptake (dotted line and crosses). Left-hand ordinates: lithium effect (%). Right-hand ordinates: lithium uptake (mM). Abscissae: lithium concentration in bathing medium (A) and time of exposure (B). Each point gives the mean and the bars  $\pm 1$  s.e. of results from four batches of embryos. The lithium effect is taken as the sum of the percentage abnormal and percentage dead embryos after taking account of spontaneous death ( $8 \pm 2$  %) in controls. (C) Histogram giving the relation between lithium uptake and percentage lithium effect defined as in A and B. Bars give  $\pm 1$  s.e.m. Regression line (broken line) calculated from the means. The number of batches for each point is given at the bottom of each column.

staining observed in the lithium-treated embryo with the anti-neurofilament antibody arose from neurones (unpublished observations of S. Rowe). Fig. 5B,D



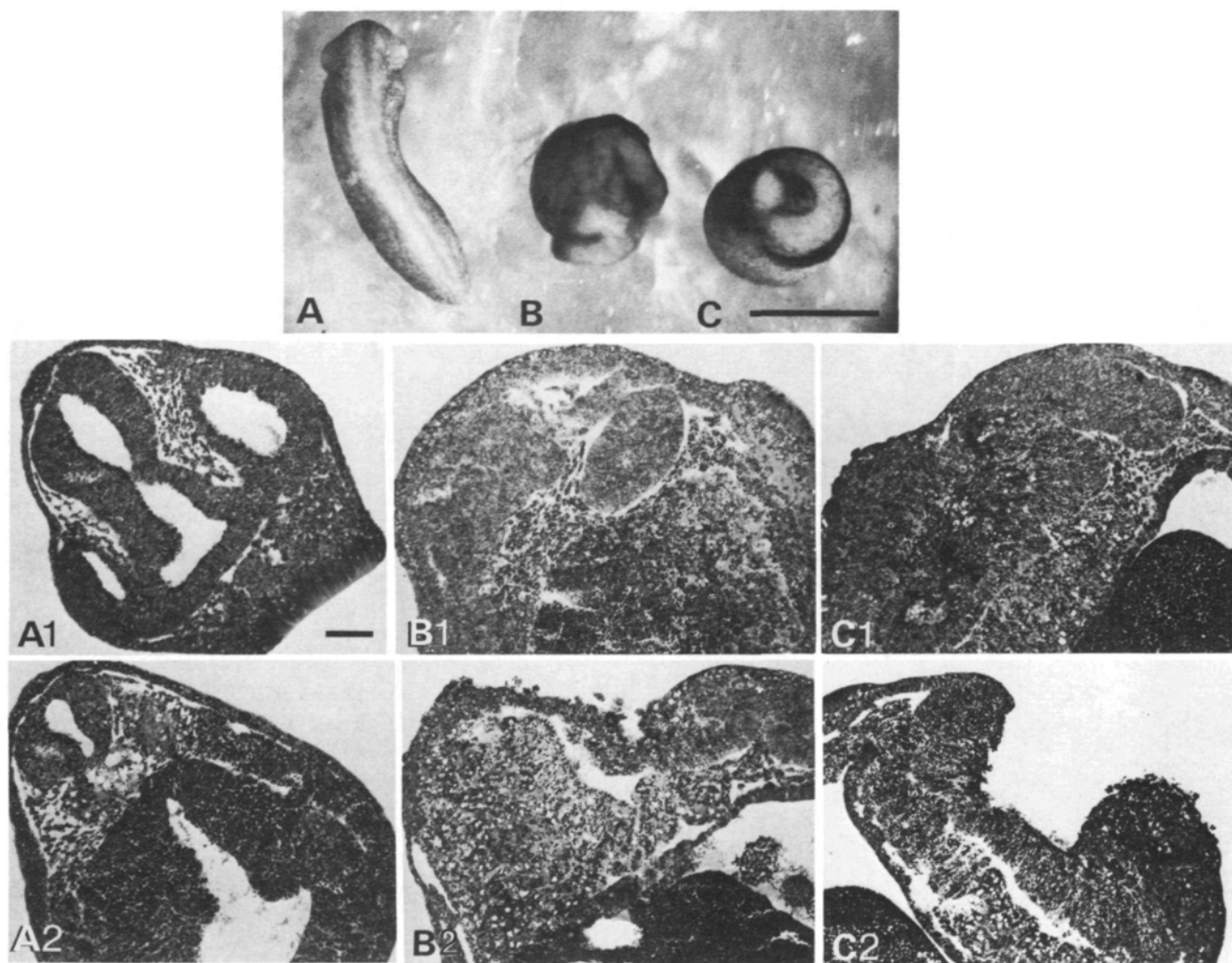
**Fig. 3.** The sensitivity to lithium declines with stage of development. (A) The teratogenic effect of 4 h exposure to lithium initiated at progressively later stages of development. Ordinate: lithium effect (%). Abscissa: stage at which treatment was initiated. The hatched area within each bar gives the proportion of embryos that died as a result of treatment, the clear area the proportion of abnormal embryos. Bars give  $\pm 1$  s.e., the numbers in parentheses the number of batches for each point (14 embryos per batch). Treatment initiated at the 32- to 64-cell state and later was significantly less effective than treatment beginning at the 2- to 4-cell stage. \* $P < 0.02$ ; \*\* $P < 0.001$ . Student's *t*-test. (B) The uptake of lithium in sibling embryos at selected stages of development. Ordinate: Concentration of lithium in the embryo at the end of the treatment period. Abscissa: stage at which treatment initiated. Bars  $\pm 1$  s.e. Number of batches for each point in parentheses, four to six embryos per batch. Note lithium uptake does not change significantly with age.

shows the pattern of muscle cell differentiation recognized with an anti-muscle monoclonal antibody (12/101: Kintner & Brockes, 1984) in the trunk region of a control (B) and a lithium-treated embryo (D). In the control embryo, myotomal muscle cells flank the notochord (see also Gurdon, Mohun, Brennan & Cascio, 1985). By contrast, the lithium-treated embryo contained groups of disorganized axial muscle cells. Six lithium-treated embryos, showing varying degrees of internal disorganization, all gave positive staining with both antibodies. These results show that both neurones and muscle cells differentiate in lithium-treated embryos, despite the gross disruption of pattern formation.

#### Quantitative assessment of neuronal differentiation

Although differentiated neurones can still be recognized in embryos treated with lithium, it is possible that the amount of neural differentiation is reduced as compared with controls. If the hypothesis that lithium inhibits neural induction is correct, then absolute levels of neural differentiation ought to be reduced in embryos previously treated with lithium. To test this possibility we compared neural differentiation in monolayer cultures prepared from the neural tube and somitic mesoderm of control embryos at stage 20, just before outgrowth from the neural tube begins, and from the dorsal surfaces of abnormal, lithium-treated embryos of the same age. 18 h after plating, control cultures have formed a monolayer of differentiated cells in which neurones and muscle cells are readily identified using morphological criteria (see Messenger & Warner, 1979; Breckenridge & Warner, 1982). Cells identified morphologically as neurones generate action potentials, drive contractile activity in innervated muscle cells and are specifically recognized by the anti-neurofilament antibody. Morphologically identified muscle cells generate end-plate potentials and contract when innervated, develop striations and stain with the anti-muscle antibody (Breckenridge, unpublished observations, see also Warner, 1985). The cultures also contain pigment cells, fibroblastic and epithelial cells.

The number of nerve and muscle cells, together with the total number of cells (100–500 cells/field), was counted in each of 20–30 microscope fields taken at random from three cultures prepared from control embryos or from sibling embryos previously treated with lithium during the early cleavage stages. The percentage of nerve and muscle cells was then calculated for each field and plotted as a frequency histogram. It was sometimes difficult to be certain that embryos that had been severely affected by the lithium treatment were still alive at the time of culture. To avoid this complication, the experiment was discarded when the total number of cells that



**Fig. 4.** Lithium treatment generates gross internal disorder. (A–C) Photographs of control embryo at stage 26 and two severely abnormal lithium-treated siblings of the same age. Bar, 1 mm. (A1, B1, C1) Sections taken through the head at the level of the optic cups in the control and the best estimate of the equivalent level in the lithium-treated embryos. (A2, B2, C2) Sections through the midtrunk region. Bar, 10  $\mu$ m. Note extreme internal disorder.

differentiated from lithium-treated embryos was significantly ( $P < 0.01$ ) less than from controls.

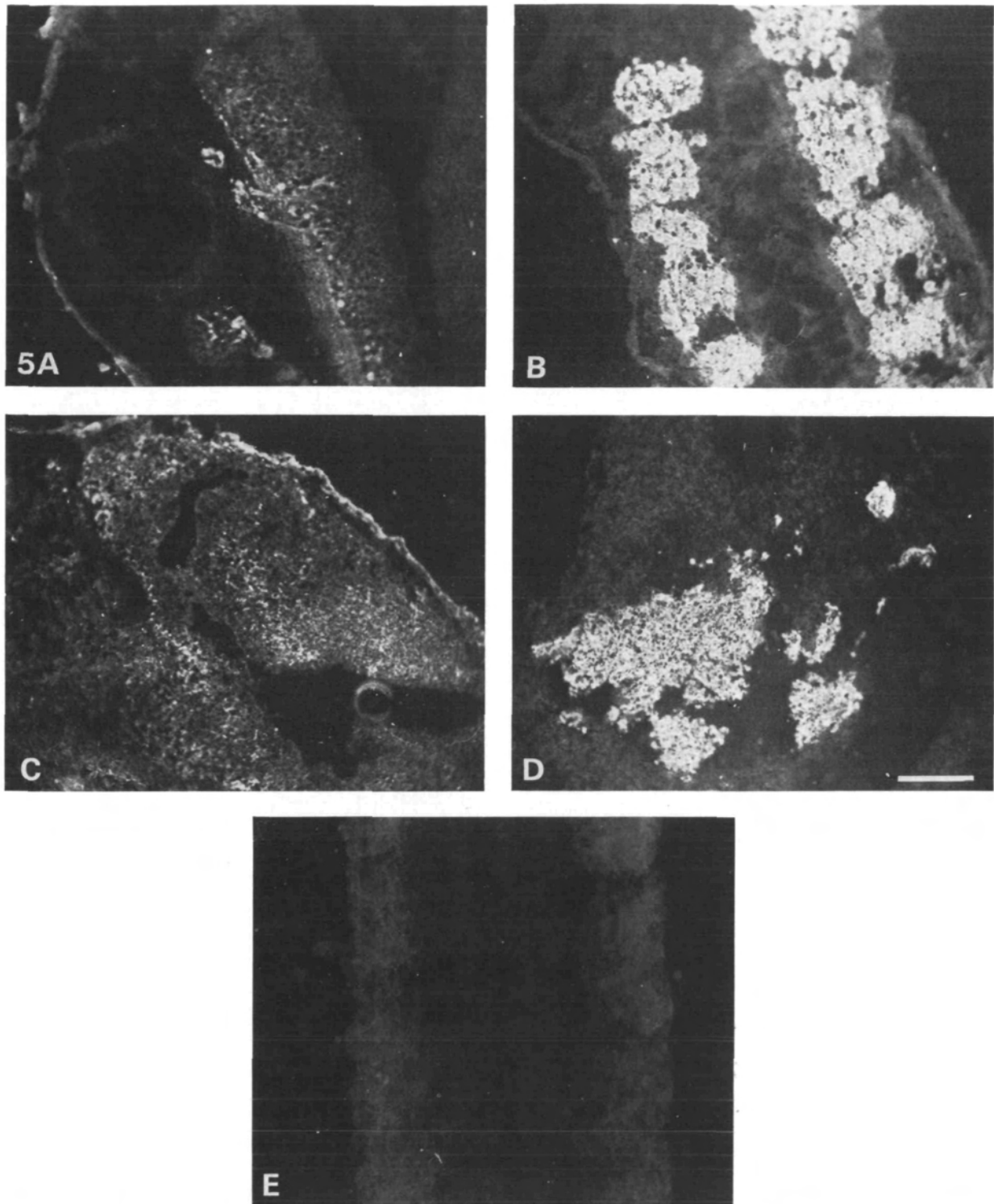
Fig. 6 shows frequency histograms of the proportion of nerve cells differentiating from control embryos (A) and from embryos previously treated with 100 mM-LiCl from the 2- to 4-cell stage (B) for one of these experiments. Total cell numbers were not affected by the lithium treatment, suggesting that all the embryos were alive at the time of culture. The median of the control distribution is at 3.9%. The number of neurones differentiating from the abnormal, lithium-treated embryos was substantially greater, with the median of the distribution at 8.2%. The two distributions are significantly different ( $P < 0.001$ ; Mann–Whitney test). By contrast, the proportion of muscle cells differentiating from normal and lithium-treated embryos was unaffected. Fig. 6C shows the average proportion of neurones per

field in eight experiments in which both total cell numbers and the degree of muscle cell differentiation were unaffected by lithium treatment. It is clear that lithium treatment during the early cleavage stages substantially increases the proportion of neurones that subsequently differentiate.

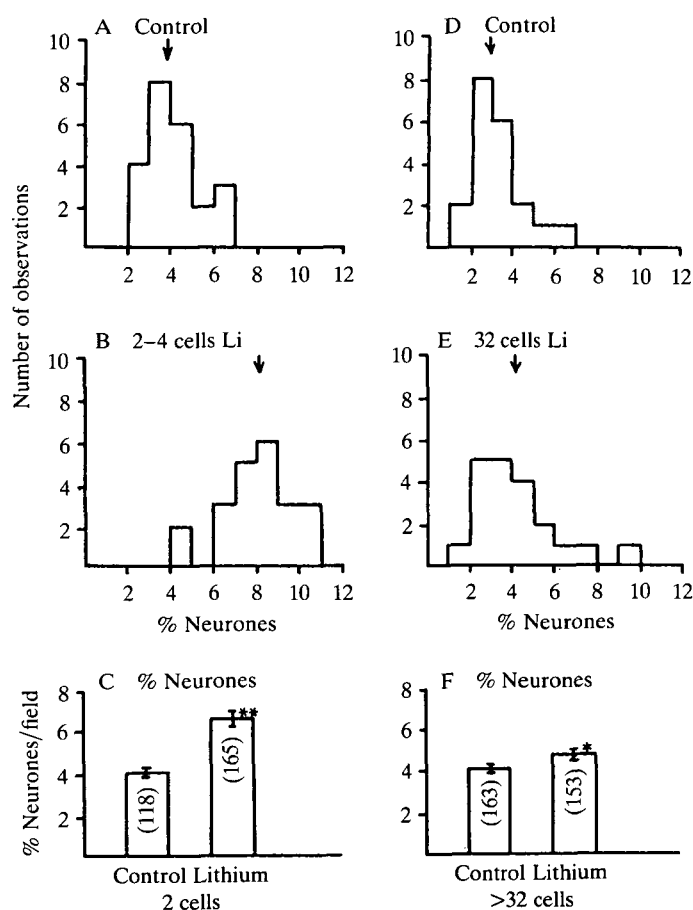
Taken together, these two sets of experiments make it unlikely that lithium treatment during the early cleavage stages inhibits neural induction. They also show that judgements based entirely on the external appearance of the embryo may be misleading. Failure of the morphogenetic movements of neurulation and the absence of normal neural patterning do not necessarily imply that phenotypic differentiation of neurones has also been prevented.

The ability of lithium treatment to generate abnormalities declines with increasing embryonic age. Fig. 6D,E compares the proportion of neurones that





**Fig. 5.** Cell-type-specific antibodies recognize nerve and muscle cells even in severely disorganized, lithium-treated embryos. Sagittal frozen sections through control embryo at stage 28 (A,B) and lithium-treated embryos of same age (C, D). A and C stained with antibody directed against neurofilament protein. Note labelling of white matter at periphery of developing brain and the otic nerve in the control. Neural staining is widely scattered and disorganized in the lithium-treated embryo. B and D stained with antibody that recognizes axial muscle. Note clear staining of myotomal muscle in the control and the scattered patches of labelled muscle cells in the lithium-treated embryo. (E) Section through control embryo treated with second layer antibody alone. There is no background staining. Bar, 100  $\mu\text{m}$ .



**Fig. 6.** Neuronal differentiation after lithium treatment assayed quantitatively in tissue culture. (A) Frequency distribution of the percentage of neurones in each field in cultures prepared from control embryos. (B) Similar distribution for neuronal differentiation from embryos treated for 4 h with 100 mM-lithium from the 2- to 4-cell stage. Ordinates: number of observations. Abscissae: % neurones per field. (C) Pooled data from eight similar experiments. Ordinate: % neurones/field. Arrows at the median of each distribution. Bars give mean  $\pm$  1 s.e. Total number of fields counted given in parentheses. (D-F) A similar set of plots for an experiment where lithium treatment for 4 h began at the 32-cell stage. \* $P < 0.01$ ; \*\* $P < 0.001$ .

differentiated from control embryos and from embryos treated with lithium for 4 h from the 32-cell stage. The lithium-treated embryos showed minor abnormalities of the type illustrated in Fig. 1B,C. The median is 3.9% for the control population and 3.5% for the lithium-treated population. The two distributions are not significantly different ( $P > 0.1$ ; Mann-Whitney test). The pooled data from eight experiments in which treatment with lithium began between the 32-cell stage and stage 11 is shown in Fig. 6F. The proportion of neurones differentiating from lithium-treated embryos is slightly higher than found in controls but is barely significant ( $0.02 >$

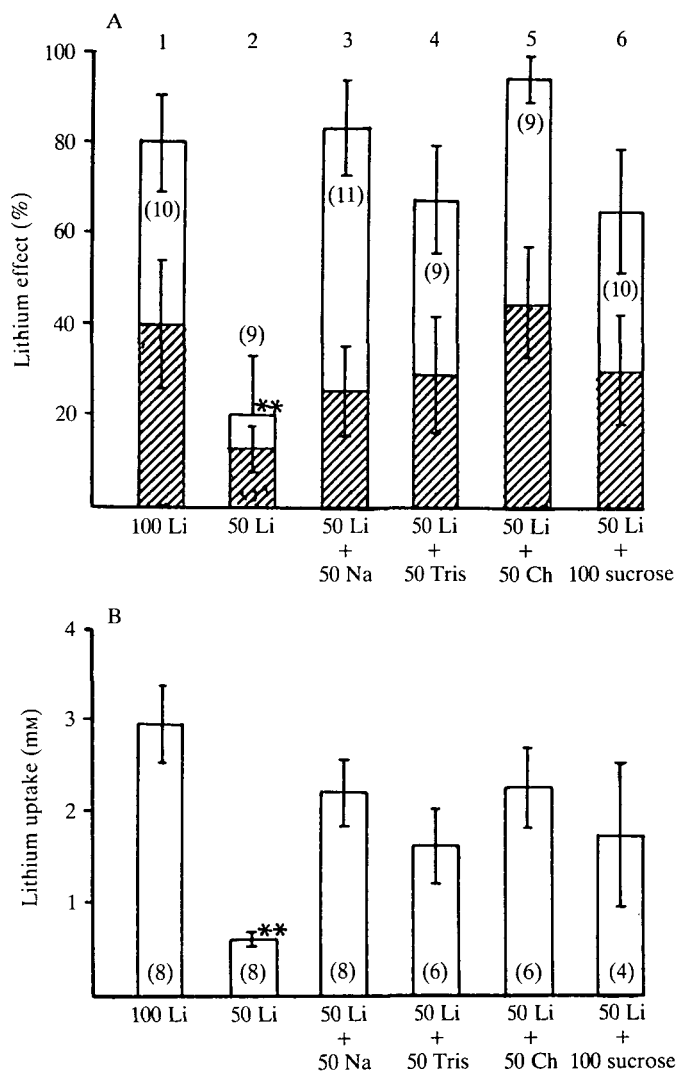
$P > 0.01$ ). There was no effect on either total cell number or the proportion of differentiated muscle cells. These results suggest that the increase in neuronal differentiation observed in embryos treated with lithium from the 2-cell stage is linked with the severity of the abnormalities.

#### Factors determining the uptake of lithium

In the course of experiments to examine the mechanism of lithium uptake by the amphibian embryo we made a number of observations on the conditions that determine the ease of entry of lithium ions. Although these experiments were not able to shed any light on the mechanisms underlying the teratogenic effects of lithium, they nevertheless provide information that may be useful for those who wish to design future experiments and are therefore described briefly below.

#### The effect of ionic strength

The lithium uptake by treated embryos increased fivefold for a doubling of the external concentration of lithium from 50 to 100 mM. This implies that the entry of lithium is not simply determined by the sum of the electrical (the membrane potential) and chemical (the concentration gradient) inward driving forces and that the permeability to lithium must have risen. To determine whether this increase in lithium permeability was induced by lithium itself or by the rise in ionic and osmotic strength of the bathing medium, we examined the uptake of lithium from 50 mM-LiCl when the ionic or osmotic strength of the external solution had been increased to that of 100 mM-salt by the addition of other compounds. Fig. 7 shows the teratogenic effect of 50 mM-LiCl (A) and the corresponding lithium uptake (B) under these conditions. As in all previous figures the numbers in parentheses give the number of batches used for each set of measurements and the values in panel A take account of spontaneous death (19% for this series). Columns 1 and 2 in each panel show the effect of 100 mM- and 50 mM-LiCl. The other columns give the effect of 50 mM-LiCl together with 50 mM-NaCl (3), Tris buffer (4) or choline chloride (5). Column 6 shows the consequences of adding 100 mM-sucrose to 50 mM-LiCl, to raise the osmotic, but not the ionic, strength. In all cases both the teratogenic effect and the uptake of lithium from 50 mM-LiCl were increased to the level normally seen after treatment with 100 mM-lithium. The permeability of the embryo to lithium must, therefore, depend on the osmotic strength of the medium bathing the embryo. The increase in permeability induced by raising osmotic strength may extend to all ions and small molecules. Loeffler & Johnston (1964) found that the uptake of tritiated thymidine by urodele embryos could be increased by



**Fig. 7.** Raising the osmotic or ionic strength increases the defects generated by lithium (A) and the lithium uptake (B). (A) Each column gives the sum of the percentage abnormal embryos (clear) and the percentage of embryos that died as a result of treatment (hatched). Bars  $\pm 1$  s.e. The treatment schedule is given at the bottom of each column. Number of batches used given in parentheses. Treatment with 50 mM-lithium alone is significantly less effective (\*\* $P < 0.001$ ) than all solutions at double the ionic/osmotic strength. All the cations and sucrose increase the effect of lithium equivalently. (B) The lithium uptake in some embryos from a number of batches (given in parentheses). Uptake from 50 mM-lithium alone significantly lower than in other conditions tested.

the addition of salt to the solution bathing the embryo.

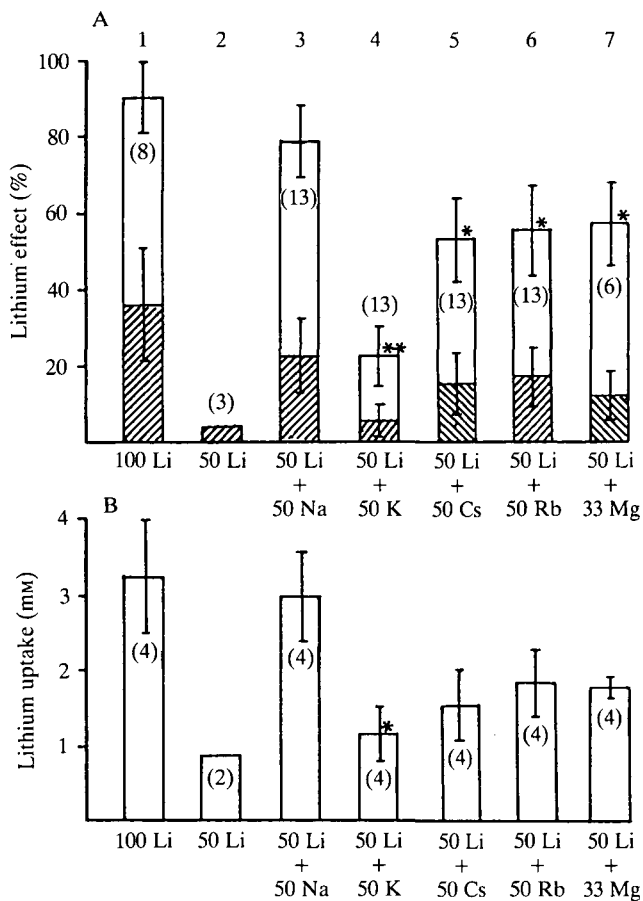
The increase in overall permeability induced by 100 mM-salt could reflect a change in the properties of the outermost cell membranes. An equivalent effect would be produced if high osmotic strength solutions interfered with the integrity of the tight junctions that

link the apical edges of each cell and are of very high resistance (Slack & Warner, 1973; see also Regen & Steinhardt, 1986), so allowing direct access to the internal, more permeable and potassium-selective cell membranes (Woodland, 1968; Slack & Warner, 1973; de Laat, Buwalda & Habets, 1974). This would explain the increased potassium sensitivity of the membrane potential of cells in embryos at high ionic strength (see below and Appendix). Electrophysiological tests to distinguish between these two possibilities are described in the Appendix. They make it most likely that high ionic or osmotic strength acts directly on the properties of the cell membranes, rather than the integrity of the tight junction. However, this conclusion may not be valid when the embryo is subjected to solutions of very high ionic strength, such as those used by Kao *et al.* (1986). If we assume that about 2.5 mM intraembryonic lithium is responsible for their finding that 6 min exposure to 0.3 M-LiCl generates similar defects to those reported here, then the permeability to lithium must have been 40 times greater than that in 50 mM-LiCl. Such a massive increase in lithium uptake could well reflect transient disruption of the properties of tight junctions, in addition to effects on cellular membranes.

#### *The cation composition of the bathing solution influences lithium uptake*

The properties of the channels opened by an increase in osmotic or ionic strength may be quite complex since the increase in lithium uptake was strongly influenced by the identity of the added ion. Fig. 8 shows the effect of adding the chlorides of potassium, caesium, rubidium or magnesium to 50 mM-LiCl compared with that of NaCl. The presence of 50 mM-potassium had little influence on either the teratogenic effect (A, column 4) or uptake of lithium (B, column 4) from 50 mM-lithium. Caesium, rubidium and magnesium all increased the abnormalities and lithium uptake in 50 mM-lithium, but were significantly less effective than sodium. Thus some cations may protect the embryo from the consequences of increasing the ionic strength.

This protection could be the consequence of competition between lithium and other cations for the sites that mediate the transfer of lithium across the outermost membranes. Alternatively sodium and lithium ions may bring about a larger increase in permeability to lithium than the other salts used to raise the ionic strength. Experiments designed to test this point directly using electrophysiological techniques are described in the Appendix. They show clearly that the overall permeability of the amphibian embryo is closely similar in a 100 mM solution of all the monovalent cations. The increase in permeability is, however, greatly reduced when the ionic strength



**Fig. 8.** Comparison of the ability of different cations to increase the teratogenicity (A) and uptake (B) of lithium. (A) Sodium and lithium added to 50 mM-lithium are equivalently effective.  $K^+$ ,  $Cs^+$ ,  $Rb^+$  and  $Mg^{2+}$  all generate a lower lithium effect than  $Na^+$ . \* $P < 0.01$ . \*\* $P < 0.001$ . (B) The lithium uptake parallels the lithium effect.

is increased by addition of the divalent magnesium ion, probably because the membrane stabilizing effect of magnesium overcomes the permeabilizing effect of high ionic strength. The most likely reason for the reduction in lithium uptake in the presence of potassium, and to a lesser extent with caesium and rubidium, is therefore direct competition between the various monovalent cations for the uptake sites.

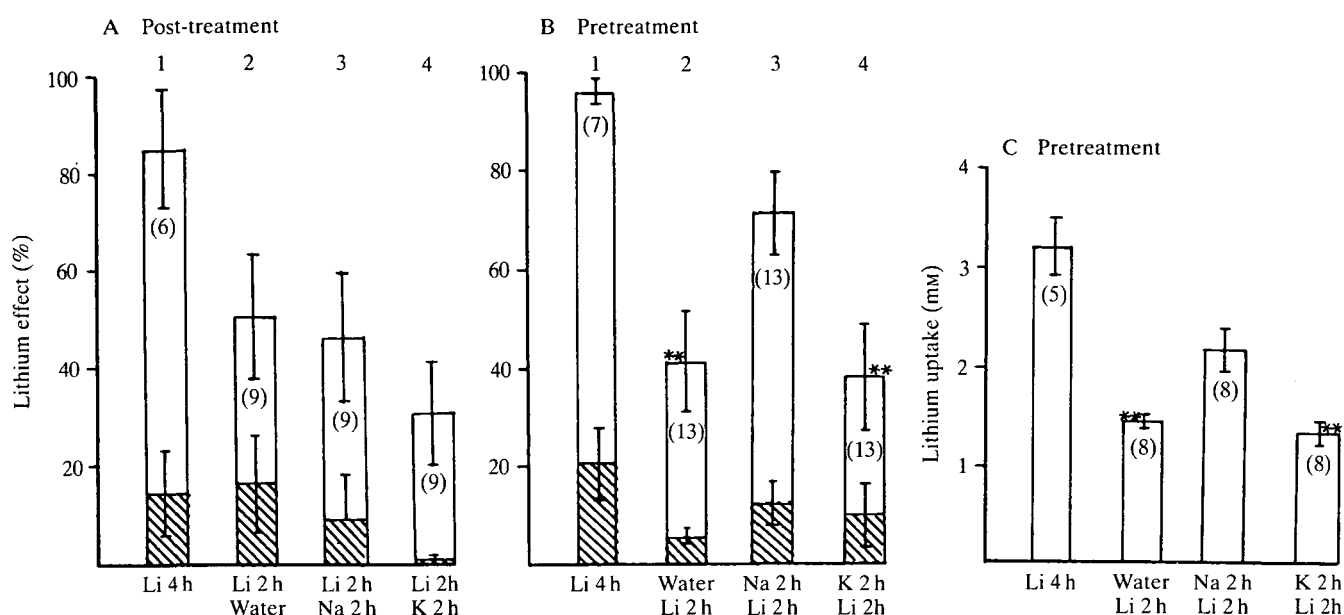
However, this cannot be the only factor. Fig. 9 shows the results of experiments in which embryos were treated with 100 mM-NaCl or KCl for 2 h either after a 2 h exposure to 100 mM-LiCl (A) or before 2 h treatment with lithium (B). The effectiveness of sodium or potassium was determined by comparison with an equivalent exposure to tap water. The salt composition of the solution bathing the embryos for the first 2 h from the 2- to 4-cell stage, that is up to the 32- to 64-cell stage, largely determines the outcome. Exposure to potassium after

lithium achieves a small, but not significant, protection of the embryos against the teratogenic effects of lithium. However, the most striking finding was that soaking the embryo in NaCl from the 2- to 4-cell stage to the 32- to 64-cell stage and then transferring the embryos to lithium potentiated both the teratogenic effects (B) and the uptake of lithium (C).

These findings imply that the ionic composition of the solution initially used to raise the ionic strength influences the properties of the channels which then become available for the movement of small molecules across the outer membranes of the embryo. We were unable to identify the nature of this influence. The only difference between the membrane properties of embryos exposed to NaCl and KCl was in the membrane potential: cells of embryos in 100 mM-lithium or in 50 mM-lithium together with sodium had resting potentials that were about 10 mV more negative than those of embryos in 100 mM-potassium or in 50 mM-lithium together with potassium. The membrane potential was also depolarized in the presence of rubidium and caesium, but to a lesser extent. However, the difference in membrane potential did not persist beyond the period of exposure to potassium ions. Embryos that had been exposed to 100 mM-potassium for 2 h promptly hyperpolarized on transfer to 100 mM-lithium.

## Discussion

Lithium ions generate teratogenic effects in the amphibian embryo at very low internal concentrations. The threshold was at about 0.5 mM, with the maximal effect being brought about by 2.5 mM-lithium. However, much of the lithium that enters the embryo moves out of the cells into the intercellular fluid, where the lithium concentration was 17 mM. Since the blastocoel fluid is probably generated by the activity of the sodium pump (Slack & Warner, 1973), lithium ions must be pumped out of the cell in place of sodium, as observed by Thomas, Simon & Oehme (1975) in snail neurones. When allowance is made for the lithium extruded into the blastocoel fluid, the maximally effective intracellular lithium concentration comes out to 0.8 mM. On the same basis, the intracellular threshold lithium concentration must be about 0.15 mM, when there would be about 3 mM-lithium in the blastocoel fluid. Thus lithium must be handled by the sodium pump more efficiently than sodium. The sodium concentration in the blastocoel is about 100 mM, while the free intracellular sodium concentration is in the region of 20 mM (Slack *et al.* 1973; Gillespie, 1983), an intercellular to intracellular ratio of 5. The equivalent ratio for lithium is nearer to 20. If some lithium is held in a bound form inside the cell then the affinity of the sodium pump for lithium



**Fig. 9.** The consequence of treating embryos with 100 mM-NaCl or 100 mM-KCl either after a 2 h exposure to 100 mM-lithium beginning at the 2- to 4-cell stage (A) or before a 2 h exposure to lithium (B). Treatment schedule given below each column. (A) There is no difference between exposure to Na<sup>+</sup>, K<sup>+</sup> or water after LiCl. (B) Sodium potentiates the teratogenic effect of a subsequent exposure to lithium. (C). Lithium uptake in embryos treated with water, NaCl or KCl for 2 h before treatment with lithium. Sodium significantly increases the subsequent uptake of lithium. \*\**P* < 0.001.

would be even higher. The free ion concentration, which may be most relevant for the teratogenic effects, could be five times lower than the estimates given above. There are no measurements of the lithium taken up by embryos in previously published experiments although the treatment schedules of Hall (1942) and Backstrom (1954) suggest very similar final lithium concentrations to those found here.

The intracellular concentration of lithium that is sufficient to generate a substantial teratogenic effect is comparable to the therapeutic dose of lithium used to treat patients with manic depressive illness (0.7–1.5 meq l<sup>-1</sup> plasma). There is full equilibration of lithium across the placental membrane (Schou & Amdeson, 1975) and lithium may also enter the uterine fluid, an ultrafiltrate of plasma. Studies on the amphibian embryo could therefore be more relevant to possible teratogenic effects in human embryos than has hitherto been realized.

The absolute permeability to lithium of the outer membranes of the amphibian embryo is normally very low, as for all other small ions (Slack *et al.* 1973; de Laat *et al.* 1974). However, the lithium uptake, and therefore the permeability, was strongly dependent on both the osmotic/ionic strength of the bathing medium. A rise in osmotic strength to that equivalent to 100 mM-salt substantially increased the uptake of lithium, probably because of a general increase in the permeability of the outer membranes of the embryo to all ions and small molecules. However, calculation

of the lithium permeability in 100 mM-lithium using the Goldman equation (see Hodgkin & Horowitz, 1959), with the influx taken from the net uptake of lithium and the membrane potential set at -30 mV, suggests that even in 100 mM-lithium the permeability coefficient is probably not much greater than  $0.04 \times 10^{-6} \text{ cm s}^{-1}$ . The sensitivity of the membrane permeability to increased ionic strength almost certainly explains why Kao *et al.* (1986) were able to generate substantial teratogenic effects by a few minutes' exposure to 0.2–0.3 M-LiCl. If the effects observed in those experiments were achieved by intraembryonic lithium concentrations similar to those measured here, then their results imply a lithium uptake at a rate at least 40 times greater than in 100 mM-LiCl, making the lithium permeability coefficient in the region of  $2 \times 10^{-6} \text{ cm s}^{-1}$ . This would suggest that the outer membrane of the *Xenopus* embryo becomes at least as permeable to small ions as frog muscle under conditions of very high ionic strength.

The external defects generated by lithium treatment in the present experiments were closely similar to those seen by others (e.g. Hall, 1942; Backstrom, 1954; Kao *et al.* 1986). They were accompanied by gross internal disorganization of all the structures of the embryonic axis, showing that pattern formation had been severely disrupted. Nevertheless differentiated neurones and differentiated muscle cells could

be identified with cell-type-specific antibodies, contradicting former suggestions that lithium ventralizes (e.g. Backstrom, 1954) or mesodermalizes (Ogi, 1961; Masui, 1961) the amphibian embryo. This conclusion is strongly supported by the observation that after lithium treatment, neuronal differentiation, assayed in tissue culture, was substantial even from acutely abnormal lithium-treated embryos. Muscle differentiation was unaffected. A further, important conclusion is that lithium has separate effects on the elements of neural induction that lead to the differentiation of nerve cells and those that lead to the patterning of neural and other axial structures.

The finding that the differentiation of neurones was significantly enhanced in lithium-treated embryos was unexpected. Although lithium has been shown to induce the differentiation of neurones from competent *Rana* ectoderm (Barth & Barth, 1974), it does not appear to do so in *Xenopus* (Messenger, 1979). One possibility is that the failure of the neural tube to close allows an inducing factor to spread further within the ectoderm (Warner, 1979). Alternatively, the suggestion of Kao *et al.* (1986) that lithium enhances dorsoanterior development could mean that the amount of neural tissue is increased. If this enhancement were extensive then it might mechanically impede closure of the neural tube. In that case the inhibition of neurulation movements would be a casual rather than a causal consequence of lithium treatment.

The finding that both lithium treatment and microinjection of lithium into vegetal cells can restore the development of dorsal structures in radially symmetric, ventral embryos generated by u.v. irradiation prior to first cleavage (Kao *et al.* 1986) provides strong support for the view that lithium enhances the development of dorsal structures rather than inhibiting neural induction. This is strengthened by Kao *et al.*'s (1986) finding that microinjection of lithium into ventral vegetal cells of normal embryos at the 32-cell stage generated a high proportion of embryos lacking posterior development, but possessing two heads. To achieve substantial rescue, or duplication of dorsal structures, Kao *et al.* (1986) microinjected 4 nl of 0.3 M-lithium in 200% Steinberg's solution into a single vegetal pole cell. If the lithium remains within the injected cell then the intracellular concentration would be in the region of 40 mM, about 10 times higher than a lethal dose. Some of the injected lithium must be distributed throughout the embryo, since our results show that most of it is destined to be extruded into the blastocoel fluid, which has access to all cells. The rate at which this redistribution of lithium takes place is not known and is difficult to estimate. At complete redistribution, the intra-embryonic lithium concentration in Kao *et al.*'s (1986)

experiments would be in the region of 2 mM, close to the concentration found by us to be maximally effective. If lithium were rapidly redistributed throughout the embryo after injection into one cell then one would expect injections into the animal or vegetal pole or into ventral or dorsal vegetal cells to be equally effective. This is not observed experimentally, suggesting that movement of lithium within the embryo is relatively slow.

An additional complication arises from the marked stage dependence of the sensitivity of the *Xenopus* embryo to lithium treatment. In the present experiments the teratogenic consequences of exposure of the embryos to lithium declined steadily as development proceeded. The lithium uptake did not decline equivalently, suggesting that some relatively early event is sensitive to the intracellular lithium concentration. The increase in neural differentiation seen in embryos exposed to lithium from the 2- to 4-cell stage also no longer took place, or was greatly reduced, when lithium treatment began at times beyond the 32-cell stage. This observation provides further evidence that the quantitative increase in the number of neurones that differentiate is directly linked to the teratogenic effects of lithium. Kao *et al.* (1986) also observed that the effects of lithium were stage dependent. Brief treatment with 0.2 or 0.3 M-LiCl was most successful in rescuing embryos from the consequence of u.v. irradiation when applied at the 32- to 64-cell stages. If the teratogenic effect of lithium is mainly determined by the intracellular concentration of lithium at the 32- to 64-cell stage, then the effective concentration must be even lower than indicated above, since total lithium was little more than 1 mM by the time embryos initially exposed to lithium chloride at the 2- to 4-cell stage had progressed through the requisite number of cleavage cycles. However, Kao *et al.*'s (1986) finding that exposure to lithium up to the 32-cell stage was substantially less effective than at the 32-cell stage makes interpretation of the stage dependence difficult. Our experiments suggest that the outer membranes of the amphibian embryo return to the low permeability state promptly (within less than 1 min) on lowering the ionic strength (see Appendix). Lithium that entered during earlier stages in Kao *et al.*'s (1986) experiments ought therefore to be present at the 32- to 64-cell stage and available to influence the putative, stage- and lithium-dependent event. However, if the increase in permeability produced by a 0.2 or 0.3 M bathing solution persists beyond the exposure, much of the lithium that enters the embryo would be immediately lost. We found that microinjection of lithium at the 2-cell stage was able to induce abnormalities. Acute stage dependence would aid the identification of the sensitive cells by microinjection

because the distribution of lithium within the embryo may be too slow for lithium ions in the appropriate cell(s) to reach threshold within the sensitive period. It may be possible to resolve some of these issues with lithium-sensitive microelectrodes.

The uptake of lithium was markedly affected by the identity of the cation used to raise the ionic strength. Most notable was the interaction between lithium and potassium, so that lithium uptake was substantially impeded when potassium ions, and to a lesser extent rubidium and caesium, were also present in the bathing medium. Lallier (1960) and Wolcott (1981) found that the teratogenic effect of lithium on sea urchin development was reduced in the presence of potassium suggesting that potassium may impede lithium entry also in the sea urchin embryo. The evidence suggests that potassium ions compete with lithium at the sites made available by the rise in osmotic strength. Whether interactions between lithium and the other monovalent cations also take place at intracellular sites is difficult to ascertain. The finding that exposing the embryo to NaCl up to the 32-cell stage increased the subsequent uptake of lithium suggests that the intracellular environment may be important. The sodium and potassium concentrations in the embryo did not change significantly in high external sodium (unpublished observations), although the naturally high concentrations and the substantial variations from embryo to embryo and batch to batch (Slack *et al.* 1973; Gillespie, 1983) could easily have obscured relatively small, yet important alterations in individuals. The internal levels of other monovalent cations may also have influenced the experiments of Kao *et al.* (1986). If in their experiments the high permeability state persisted much beyond the exposure period, then significant net loss of other small ions, which experience a much greater outward driving force than lithium, may have occurred. An additional factor would be the shrinkage on exposure to hypertonic solution and subsequent swelling on return to low ionic strength media.

The mechanism underlying the profound perturbations of development initiated by treatment of early embryos with lithium is not known. In the amphibian embryo the threshold concentration is very low, in the 100–500  $\mu\text{M}$  range, which restricts the range of possible mechanisms. In the appropriate concentration range, lithium has been reported to inhibit adenyl cyclase (Thams & Geisler, 1981) and microtubule-mediated movement of sperm flagellae (Gibbons & Gibbons, 1984). Additionally it is known that lithium interferes with a wide range of other cellular processes. The complicated interactions between lithium and the other monovalent cations focus attention on a possible physiological role of sodium

and potassium in the putative lithium-sensitive, stage-dependent event. Until this event, which appears to control the fundamental process of patterning in the amphibian embryo, is identified, the effects of lithium are unlikely to be understood.

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## Appendix

The experiments comparing the uptake of lithium when the ionic strength had been raised to 100 mM by the addition of various cations suggested that the permeability of the embryo might be much less in the presence of potassium than in sodium-containing solutions. Direct information on this problem can be obtained by measuring the electrical resistance of the embryo under the appropriate experimental conditions. This Appendix briefly describes electrophysiological experiments which address this issue. Some experiments that show that the alterations in permeability probably occur in the outermost cell membranes, rather than by disruption of the tight junctions that link the apical edges of the cells, are also included. The major conclusions drawn from the results are indicated at the appropriate positions in the main text.

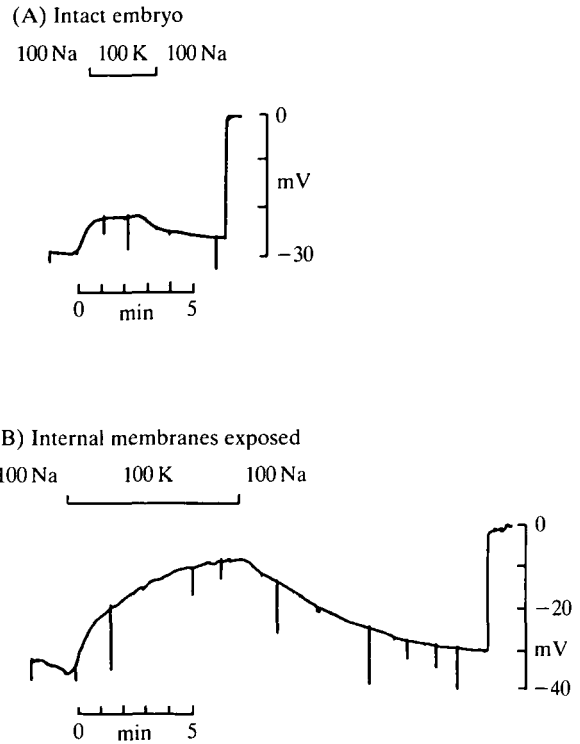
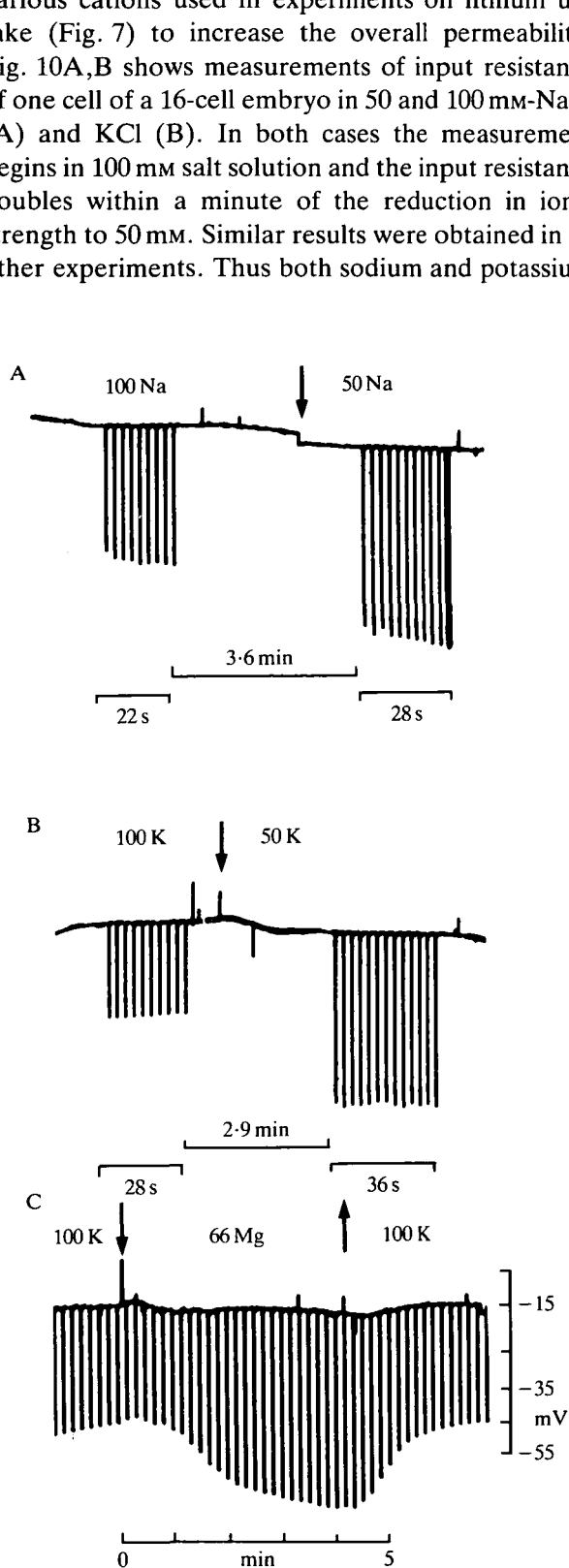
Standard electrophysiological techniques were used. Low resistance (5–10 megohm) microelectrodes filled with 3 M-KCl with insignificant tip potentials recorded the membrane potential or injected current pulses. After removing the jelly coat (either manually or by brief exposure to 2% cysteine in Holtfreter's solution at pH 8.0) the embryo was transferred to a 3 ml volume recording bath. Solution changes were effected by injecting 10 ml of solution into the bath and removing the excess with constant suction. To measure the input resistance two electrodes were inserted into the same cell, one to inject rectangular hyperpolarizing current pulses (50–300 nA, 400–1200 ms in length, one every 2–9 s) while the other recorded the membrane potential and the resultant electrotonic potentials. The ratio of the electrotonic potential to the injected current gives the input resistance of the embryo. The vitelline membrane offers a negligible resistance to the flow of current.

### *The ionic permeability at different ionic strength*

The input resistance of the cleavage-stage embryo is determined by both the properties of the low resistance, relatively potassium-permeable membranes

generated at each cell division and the high resistance, outermost membrane derived from the egg. Nevertheless, measurements of input resistance should reveal major differences in the ability of the various cations used in experiments on lithium uptake (Fig. 7) to increase the overall permeability. Fig. 10A,B shows measurements of input resistance of one cell of a 16-cell embryo in 50 and 100 mM-NaCl (A) and KCl (B). In both cases the measurement begins in 100 mM salt solution and the input resistance doubles within a minute of the reduction in ionic strength to 50 mM. Similar results were obtained in 14 other experiments. Thus both sodium and potassium

affect the overall conductance of cleavage-stage amphibian embryos equivalently. Comparison of the input resistances recorded in 100 mM-NaCl, 100 mM-LiCl and 100 mM-KCl showed the input resistance in



**Fig. 11.** The effect on the resting potential of changing from 100 mM-sodium (100 Na) to 100 mM-potassium (100 K). (A) Intact embryo. 100 mM-potassium produces an 8 mV depolarization, which is reversed on return to 100 mM-sodium. Electrode withdrawn from the cell at end of trace and the trace returns to the 0 mV baseline. (B) Vitelline membrane removed and two cells pulled apart to reveal internal membranes. 100 mM-potassium produces 30 mV depolarization. Electrode withdrawn before repolarization in 100 mM-sodium is complete.

**Fig. 10.** The input resistance of the embryo is the same in NaCl and KCl. Pen records of the membrane potential (continuous trace) and the electrotonic potential generated by injection of constant, hyperpolarizing current pulses (vertical deflections) in (A) 100 mM-NaCl (100 Na) and (B) 100 mM-KCl (100 K). For measurement of the electrotonic potential the chart speed was increased. The solution was changed to 50 mM-salt (50 Na, 50 K) as indicated and a further set of electrotonic potentials recorded. Right-hand calibration gives the resting potential of the cell. The increase in input resistance seen on halving the ionic strength is fully established within 1 min of the solution change. Na<sup>+</sup> and K<sup>+</sup> are equivalently effective. (C) Continuous record of membrane potential and the electrotonic potential in 100 mM-KCl. 66 mM-MgCl<sub>2</sub> (66 Mg) replaced KCl during part of the record. The input resistance doubles in magnesium, even though the ionic strength of the two solutions is the same.



KCl to be about 10 % less than in either NaCl or LiCl. The findings illustrated in Fig. 7 require the ionic resistance of the embryo to be much higher (by at least a factor of 2) in potassium than in sodium. Fig. 10C shows how the input resistance altered in one of seven experiments in which the bathing medium was switched from 100 mM-KCl to 66 mM-MgCl<sub>2</sub> (the equivalent ionic strength). The input resistance doubles in the presence of divalent magnesium ions; the overall ionic permeability is therefore very similar to that found in 50 mM salts of the monovalent cations. In these circumstances the uptake of lithium from 50 mM-LiCl would be expected to be little different in the presence and absence of magnesium, as observed experimentally (Fig. 7).

*Is the increase in permeability the result of disruption of tight junctions?*

Although there was little difference in the input resistance of embryos in 100 mM-NaCl, LiCl and KCl, the membrane potential always depolarized by about 10 mV when potassium ions were present. An example is shown in Fig. 11A. The membrane potential recorded from intact embryos is not usually influenced by external potassium, except during cleavage, when new, potassium-sensitive membrane is transiently exposed in the cleavage furrow (Woodland, 1968; Slack & Warner, 1973). Since the membrane potential was only sensitive to alterations in external potassium concentration at high ionic strength, it seemed possible that disruption of the tight junctions in 100 mM salt might lead to the relatively potassium-permeable inner membranes being continuously accessible to the bathing solution. However, this seems unlikely. Fig. 11B shows that when the internal membranes were deliberately exposed to the bathing solution (by removing the embryo from the vitelline membrane and gently pulling two of the surface cells slightly apart) 100 mM-potassium generated a very substantial depolarization, with the membrane potential approaching the potassium equilibrium potential (0 mV). Furthermore the input resistance of the fertilized egg was as sensitive to alterations in ionic strength as multicellular embryos. The finding that the membrane potential of cells becomes potassium sensitive at 100 mM salt suggests that the channels made available for ion transport by the increase in ionic strength may reflect the unmasking of channels originally present in the oocyte membrane which normally cease to take part in ion transport on maturation and fertilization.

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