SEQUENTIAL INDUCTION OF THE PRESUMPTIVE EPIDERMIS OF THE RANA PIPIENS GASTRULA [1]

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A study of the effects of inductors applied for various lengths of time and at various concentrations has led us to the concept of sequential induction (Barth and Barth, 1963). The competent ectoderm responds to an inductor by first forming radial nerve. Further application of the inductor results in the differentiation of spreading nerve; and with still further treatment the cells which would have formed spreading nerve are induced to form pigment cells. Two more cell types in the sequence are astrocytes and neuroglia cells.

The experiments reported in this paper were designed to test the hypothesis of sequential induction by the sequential action of two different inductors. Specifically, if we first induce presumptive epidermal cells to become determined as nerve cells, will a second inductor then induce these determined nerve cells to become pigment cells?

We also asked the question: is induction at any one step in the sequence reversible? To answer this question we first induced the cells to become determined as pigment cells and then applied a strong neural inductor to see if it could reverse the induced pigment cells back to nerve cells.

Finally, we designed some experiments to test whether or not the sequence of inductions was a necessary sequence. Can pigment cells be induced without first inducing nerve cells?

During the course of the experiments it became apparent that we were obtaining a new cell type between spreading nerve and pigment cells. These cells possess the slate gray pigment granules characteristic of pigment cells, but these granules are distributed throughout the cytoplasm rather than forming a ring of pigment. The cells remain in clumps, in contrast to the later behavior of pigment ring cells. Finally, they have thick, prominent cell membranes. Thus, with the addition of this new cell type the sequence of inductions becomes epidermis, to radial nerve, to spreading nerve, to slate gray epithelium, to pigment cells, to astrocytes to neuroglia.

Experimental Methods

Explants of the presumptive epidermis are dissected out of 6 gastrulae at Stage 11 Shumway (1940) unless otherwise stated. Six explants are treated with Versene (EDTA) to loosen the bond between the outer pigmented layer of epidermis and the inner layer. The outer pigmented layer is removed and discarded. The inner layer then is divided into about 25 small aggregates of cells.

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These 150 aggregates then are transferred to solutions containing the inductors and finally to small stender dishes containing our standard salt solution, which also contains globulin from serum (Bios Laboratories, Inc., 17 W. 60th St., New York 23). An adequate substitute for Bios globulin has been found to be lyophilized calf serum (Nutritional Biochemicals Corp.). The method is the

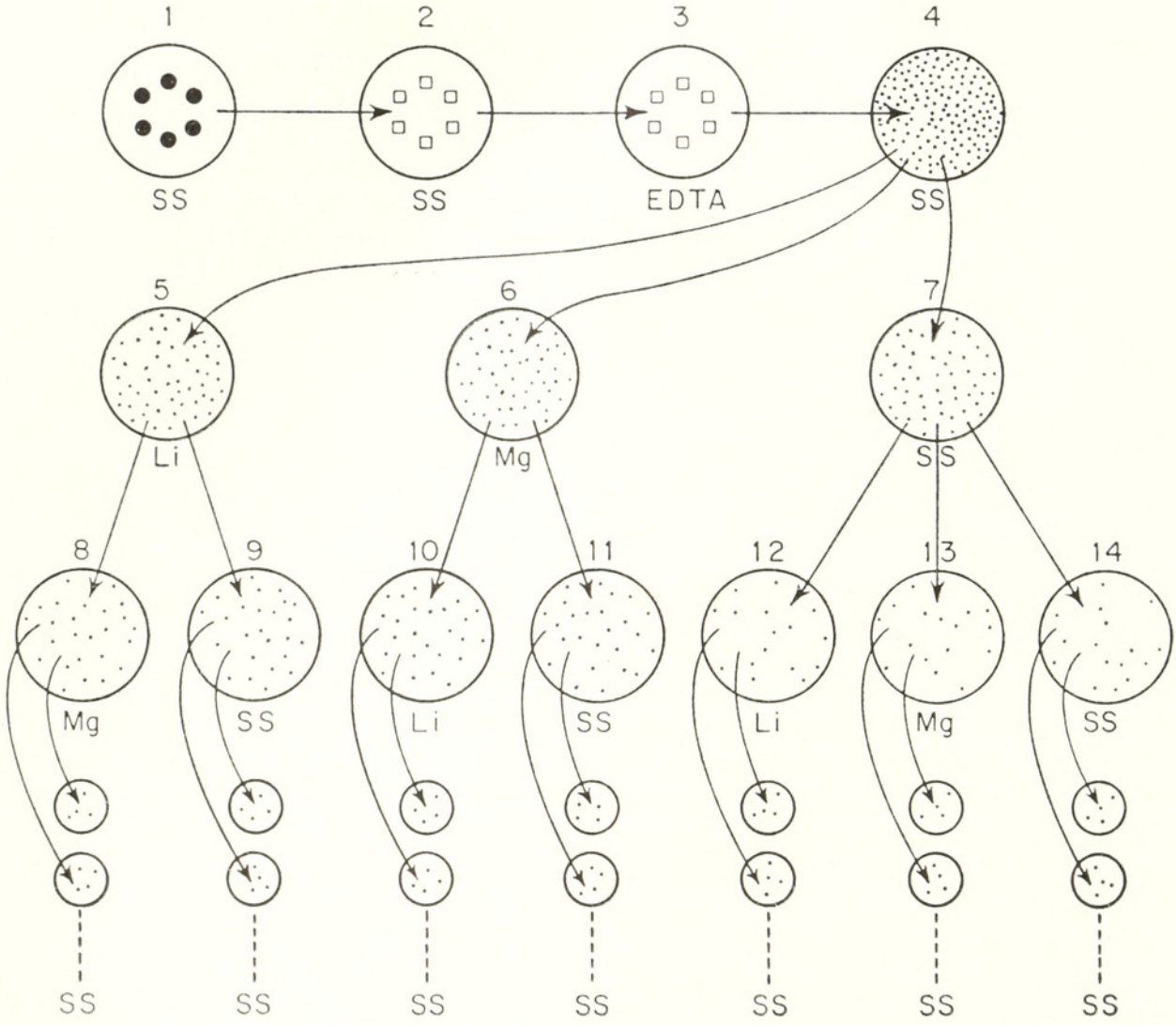

FIGURE 1. Diagram of a typical experiment involving the use of two inductors in sequence. Vitelline membranes are removed in dish 1 containing standard solution (SS). Composition of this solution is given in Barth and Barth (1959). Explants are dissected out in dish 2. Versene-treatment in dish 3 loosens bond between outer pigmented layer of epidermis and inner layer of cells. In dish 4 outer pigment layer is removed and discarded, and explants are teased into aggregates. Subsequent transfers of aggregates, all made by means of Spemann pipette, are described in the text. Abbreviations used are as follows: Li: lithium chloride; Mg: $MgSO_4 \cdot 7\,H_2O$.

same as described by Barth and Barth (1959) except that we do not use agar to coat our operating dishes. This is because agar was found to be a neural inductor (Barth and Barth, 1963).

A diagram of a typical experiment involving the use of two inductors in sequence is presented in Figure 1. The experiment diagrammed gives the following information.

1. Transfers from 4 to 7 to 14 serve as a control for differentiation of untreated epidermal cells. Such cells usually form a sheet of epithelium with ciliated patches or a mass of free-swimming ciliated cells. The control is necessary because sometimes for unknown reasons a small amount of nerve develops in controls.

2. Transfers from 4 to 6 to 10 give the sequential effect of Mg followed by Li, which results in extensive pigment cells. Two controls for this effect are (a) 4 to 6 to 11, which shows that Mg alone does not induce pigment cells, and (b) 4 to 7 to 12, which gives the effect of Li without previous treatment with Mg—usually a few scattered pigment cells or none at all.

3. Transfers from 4 to 5 to 8 show the reversibility of the Li induction by Mg. Usually no pigment cells differentiate. Transfers 4 to 5 to 9 give the results of Li alone, where some pigment cells differentiate. Sequential transfers from 4 to 7 to 13 show that Mg is still able to induce nerve cells.

Several preliminary experiments of the type outlined above showed that Mg would induce nerve, which was then induced to pigment cells by Li. However, Li, which induced pigment cells, followed by Mg resulted in no pigment cells but only nerve cell differentiation. More detailed experiments in which the times of treatment and the concentrations of two inductors were varied are given in the experimental results.

Experimental Results

Sequential action of lithium chloride after pretreatment with magnesium sulfate

In these experiments we attempted to achieve a situation by which a minimal or subminimal exposure to lithium chloride would result in a few pigment cells or none at all. Some of the aggregates were first treated with magnesium sulfate, which would induce the cells to become nerve. Other aggregates were exposed to the standard solution for the same length of time as those which were exposed to magnesium sulfate. Other controls consisted of treatment with magnesium sulfate without subsequent treatment with lithium chloride; and controls for the standard solution, *i.e.*, no treatment with either inductor but the same number of transfers at the same times.

Table I records the sequential action of magnesium sulfate followed by lithium chloride. In this and the succeeding tables the table headings are to be interpreted as follows. Stage no.: Shumway (1940); conc.: concentration of added substances in milligrams per milliliter of standard solution; time: time in hours during which the aggregates are exposed to the substances indicated; types of cellular differentiation: as described in Barth and Barth (1963) and Barth and Barth (1962).

Experiments 1–5 show that lithium chloride alone induces only a few pigment cells while most of the cells form nerve or epithelium. If the explant were first treated with magnesium sulfate for 2.5 to 3.5 hours, which is sufficient for the induction of nerve, lithium chloride induces most of the cells to differentiate into pigment cells and spreading nerve. Thus, magnesium sulfate, which is unable to induce pigment cells by itself, can induce nerve cells, which are then further induced by lithium chloride to become pigment cells.

Experiments 7 and 8 show sequential action with regard to the type of nerve induced. It is seen that magnesium sulfate induces radial nerve. If it is followed

by increasing exposures to lithium chloride the radial nerve is induced to the spreading nerve pattern of differentiation. In these experiments no pigment cells are induced because the treatments begin at early stage 11 and the cells are not competent for pigment cell induction at this stage with the times of exposure to lithium used in the experiments (Barth, 1964).

TABLE I

Sequential action of magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$) and lithium chloride ($LiCl$)

Exp. no.	Stage no.	Mg		Li		Number of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	6.0	3.0	2.0	2.0	75	Extensive pigment cells, spreading nerve
	11	0.0	3.0	2.0	2.0	75	Epithelium, few pigment cells, little nerve
2	11	6.0	2.5	2.0	3.0	75	Extensive pigment cells, nerve
	11	0.0	2.5	2.0	3.0	75	Epithelium, few pigment cells, little nerve
3	11	6.0	3.0	2.0	2.0	75	Extensive pigment cells, little nerve
	11	0.0	3.0	2.0	2.0	75	Epithelium, few pigment cells, little nerve
4	11	6.0	3.0	2.0	2.0	50	Extensive pigment cells, spreading nerve
	11	0.0	3.0	2.0	2.0	50	Epithelium, few pigment cells, some nerve
	11	6.0	3.0	0.0	2.0	25	Nerve
	11	0.0	3.0	0.0	2,0	25	Epithelium
5	11	6.0	3.5	2.0	2.0	40	Extensive pigment cells, spreading nerve
	11	0.0	3.5	2.0	2.0	35	Nerve, few pigment cells, ciliated cells
6	11–	6.0	2.5	2.0	2.0	40	Slate gray epithelium, some nerve
	11–	0.0	2.5	2.0	2.0	35	Spreading nerve, some slate gray epithelium
7	11–	6.0	2.3	3.0	0.5	15	Radial nerve
	11–	0.0	2.3	3.0	0.5	15	Epithelium
	11–	6.0	2.3	3.0	1.0	15	Spreading nerve, some radial nerve
	11–	0.0	2.3	3.0	1.0	15	Some nerve, some epithelium
	11–	6.0	2.3	3.0	1.5	15	All spreading nerve
	11–	0.0	2.3	3.0	1.5	15	Radial nerve, some spreading nerve
8	11–	6.0	2.0	4.3	0.5	25	Spreading nerve
	11–	0 0	2.0	4.3	0.5	25	Radial nerve
	11–	6.0	2.0	4.3	1.0	25	Spreading nerve
	11–	0.0	2.0	4.3	1.0	25	Radial nerve

Experiment 6 gives results intermediate between those of 1–5 and 7 and 8. Early stage 11 is used and the cells are not competent yet for pigment cell induction. Lithium chloride induces mostly spreading nerve and a few slate gray cells of a type obtained many times with lithium chloride when applied to early stage 11 cells. However, after pretreatment of the preparations with magnesium sulfate, lithium chloride induces many more slate gray cells and less nerve appears. These slate gray cells resemble those of the adrenal medulla in staining

properties, but have not been positively identified as such. In any case these cells form a step in the sequence between spreading nerve and pigment cells and they possess the same type of slate gray granules as do pigment cells.

Sequential action of calcium chloride followed by lithium chloride

Table II, experiment 1, records the data supporting the conclusion that a pretreatment with calcium chloride permits lithium chloride at minimal exposure to induce extensive pigment cells. Experiment 2 shows that increasing exposure to lithium chloride induces the nerve induced by calcium chloride to differentiate into spreading nerve and finally slate gray epithelium. In experiment 3, when a longer exposure to lithium chloride results in extensive pigment cells, pretreatment with calcium chloride gave not only extensive pigment cells but also some astrocytes.

TABLE II

Sequential action of $CaCl_2 \cdot 2\ H_2O$ and LiCl

Exp. no.	Stage no.	Ca		Li		Number of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	2.5	2.1	4.2	1.1	75	Extensive pigment cells, some spreading nerve
	11	0.0	2.1	4.2	1.1	75	Epithelium, some pigment cells, little nerve
2	11 –	2.5	2.6	4.2	0.3	25	Spreading nerve, radial nerve
	11 –	0.0	2.6	4.2	0.3	25	Epithelium, little nerve
	11 –	2.5	2.6	4.2	0.7	25	Spreading nerve, little radial nerve
	11 –	0.0	2.6	4.2	0.7	25	Radial nerve, epithelium
	11 –	2.5	2.6	4.2	1.0	25	Spreading nerve, slate gray epithelium
	11 –	0.0	2.6	4.2	1.0	25	Radial nerve, spreading nerve, few slate gray cells
3	11	2.5	3.2	3.0	2.0	60	Extensive pigment cells, some astrocytes, some nerve
	11	0.0	3.2	3.0	2.0	60	Extensive pigment cells, some nerve

From all the experiments in Table II we conclude that lithium chloride appears to carry on the sequence of inductions from radial nerve to spreading nerve to slate gray cells to pigment cells and finally to astrocytes.

Combination of the action of an inductor followed by culture in a low concentration of lithium chloride

Continuous culture in low concentrations of lithium chloride in the standard solution brings about the induction of astrocytes and neuroglia cells. At a concentration of 0.40 mg./ml. astrocytes form; at 0.47 to 0.65 mg./ml. neuroglia differentiate. If we first induce the cells to spreading nerve or pigment cells and then culture in low concentrations of lithium chloride what cell types would we obtain?

TABLE III

Combination of the actions of an inductor and a low concentration of LiCl in the culture solution. Li:LiCl; Mg: $MgSO_4 \cdot 7\ H_2O$; Ca: $CaCl_2 \cdot 2\ H_2O$.

Exp. no.	Stage no.	Treatment		Culture	No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.			
1	11	3.0 Li	2.0	.00 Li	50	Extensive pigment cells
	11	3.0 Li	2.0	.15 Li	50	Extensive pigment cells, some astrocytes
	11	3.0 Li	2.0	.30 Li	50	Extensive neuroglia cells
2	10+	6.0 Mg	3.0	.10 Li	35	Spreading nerve, radial nerve
	10+	0.0 Mg	3.0	.10 Li	35	Ciliated masses, mucus
	10+	6.0 Mg	3.0	.30 Li	48	Some large, unidentifiable cells
	10+	0.0 Mg	3.0	.30 Li	48	Ciliated masses, mucus
3	11−	6.0 Mg	4.0	.10 Li	35	Spreading nerve
	11−	0.0 Mg	4.0	.10 Li	35	Ciliated epithelium, voluminous mucus
	11−	6.0 Mg	4.0	.30 Li	40	Large, unidentifiable cells
	11−	0.0 Mg	4.0	.30 Li	40	Ciliated epithelium, voluminous mucus
4	11	6.0 Mg	3.0	.20 Li	25	Loose cells, some ciliated masses
	11	6.0 Mg	3.0	.00 Li	25	Spreading nerve, radial nerve
5	11	6.0 Mg	3.0	.10 Li	35	Spreading nerve, radial nerve
	11	6.0 Mg	3.0	.05 Li	40	Spreading nerve, radial nerve
	11	6.0 Mg	3.0	.00 Li	35	Spreading nerve, radial nerve
	11	0.0 Mg	3.0	.10 Li	30	Ciliated epithelium
6	11	2.5 Ca	3.0	.10 Li	35	Spreading nerve
and	11	0.0 Ca	3.0	.10 Li	35	Ciliated masses
7	11	2.5 Ca	3.0	.30 Li	40	Loose cells, no differentiation
	11	0.0 Ca	3.0	.30 Li	40	Ciliated masses, voluminous mucus

In Table III, experiment 1, lithium chloride is first used to induce pigment cells and then the aggregates are cultured in 0.15 and 0.30 mg./ml. of lithium chloride. Astrocytes appear at 0.15 mg./ml. and neuroglia at 0.30 mg./ml. Lithium chloride in these low concentrations has little effect on aggregates, as seen from experiment 3. Pretreatment with lithium chloride to induce pigment cells permits the further induction to astrocytes and neuroglia.

The remainder of the table, experiments 2–7, records attempts to obtain inductions with low concentrations of lithium chloride after inducing the cells with magnesium sulfate or calcium chloride. It is clear that either calcium chloride or magnesium sulfate will induce the spreading nerve stage, but that subsequent treatment with 0.10 mg./ml. of lithium chloride has no effect. Subsequent treatment with 0.30 mg./ml. of lithium chloride has a deleterious effect with many loose, dead cells and only a few large, unidentifiable cells remaining alive. These results, when compared with those of experiment 1, lead us to conclude that the induction of pigment cells is a necessary step toward the induction of astrocytes and neuroglia cells. Since neither calcium chloride nor magnesium sulfate is

able to induce pigment cells, further induction by low concentrations of lithium chloride is blocked.

Reversibility of the induction: induction of pigment cells by lithium chloride followed by a neural inductor

In Table IV experiments 1–4 record data which are interpreted to mean that either the induction of pigment cells is reversible or that pigment cells cannot differentiate after treatment with magnesium sulfate. Experiment 3 in particular shows that lithium chloride alone induces pigment cells and magnesium sulfate alone induces nerve cells. When the two are applied in sequence with lithium chloride first, only nerve cells are induced.

In experiment 5 lithium chloride induced slate gray epithelium and, when further treated with magnesium sulfate, the cells formed only spreading nerve.

TABLE IV

Reversibility of the induction of pigment cells Li:LiCl; Mg: $MgSO_4 \cdot 7\ H_2O$

Exp. no.	Stage no.	Li		Mg		No. of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	3.0	2.0	6.0	3.3	75	Spreading nerve, few slate gray cells
	11	3.0	2.0	0.0	3.3	75	Extensive pigment cells, nerve
2	11	3.0	1.5	6.0	2.0	50	Spreading nerve
	11	3.0	1.5	0.0	2.0	50	Few pigment cells, spreading nerve
3	11	3.0	2.0	6.0	3.0	40	Nerve
	11	3.0	2.0	0.0	3.0	40	Pigment cells, nerve
	11	0.0	2.0	6.0	3.0	35	Nerve
	11	0.0	2.0	0.0	3.0	35	Epithelium
4	11−	3.0	3.5	6.0	2.8	40	Spreading nerve
	11−	3.0	3.5	0.0	2.8	40	Few pigment ring cells, spreading nerve
5	11−	3.0	2.5	6.0	3.0	40	Spreading nerve
	11−	3.0	2.5	0.0	3.0	35	Nerve, slate gray epithelium
	11−	0.0	2.5	6.0	3.0	40	Nerve
	11−	0.0	2.5	0.0	3.0	35	Epithelium
6	11	4.0	2.0	7.0	3.0	60	Spreading nerve, slate gray epithelium
	11	4.0	2.0	0.0	3.0	60	Pigment cells, nerve
7	11	4.0	2.0	7.0	3.0	75	Spreading nerve, slate gray epithelium
	11	4.0	2.0	0.0	3.0	75	Pigment cells, nerve
8	11+	3.0	2.0	6.0	3.5	75	Extensive pigment cells, nerve
	11+	3.0	2.0	0.0	3.5	75	Extensive pigment cells, epithelium
9	11+	2.0	3.0	6.0	2.5	70	Extensive pigment cells, nerve
	11+	2.0	3.0	0.0	2.5	70	Extensive pigment cells, nerve

In experiments 6 and 7, on the other hand, after lithium chloride induced pigment cells, magnesium sulfate reversed this induction to slate gray epithelium.

The last two experiments, 8 and 9, were performed to distinguish between reversibility of induction of pigment cells and failure of pigment cells to differentiate. By using a later stage in development (stage 11 plus) extensive pigment cells are induced by a two- to three-hour period of exposure to lithium. Subsequent treatment with magnesium sulfate has no effect on the differentiation of the

TABLE V

Reversibility of the induction of pigment cells Li:LiCl; Mg:$MgSO_4 \cdot 7\ H_2O$; Bi: $NaHCO_3$

Exp. no.	Stage no.	Treatment		Culture	No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.			
1	11–	3.0 Li	3.5	3.0 Mg	40	Spreading nerve
	11–	3.0 Li	3.5	0.0 Mg	35	Pigment cells, spreading nerve
2	11	4.0 Li	2.0	4.0 Mg	75	Spreading nerve
	11	4.0 Li	2.0	0.0 Mg	75	Pigment cells, spreading nerve
3	11	4.0 Li	2.0	4.0 Mg	50	Nerve, slate gray epithelium
	11	4.0 Li	2.0	0.0 Mg	50	Pigment cells, nerve
4	11	3.0 Li	2.5	3.0 Mg	40	Nerve
	11	3.0 Li	2.5	0.0 Mg	35	Pigment cells, nerve
	11	0.0 Li	2.5	3.0 Mg	40	Nerve
	11	0.0 Li	2.5	0.0 Mg	35	Epithelium
5	11	3.0 Li	2.0	1.0 Bi	35	Nerve
	11	3.0 Li	2.0	0.0 Bi	35	Pigment cells, nerve
	11	3.0 Li	4.5	1.0 Bi	40	Dead cells
	11	3.0 Li	4.5	0.0 Bi	40	Pigment cells, nerve, dead cells
6	11	3.0 Li	1.1	1.0 Bi	20	Nerve
	11	3.0 Li	1.1	0.0 Bi	20	Nerve, rare pigment cells
	11	0.0 Li	1.1	1.0 Bi	20	Nerve
	11	3.0 Li	2.0	1.0 Bi	20	Nerve
	11	3.0 Li	2.0	0.0 Bi	20	Extensive pigment cells
	11	0.0 Li	2.0	1.0 Bi	20	Nerve
	11	3.0 Li	3.5	1.0 Bi	20	Few pigment cells, nerve
	11	3.0 Li	3.5	0.0 Bi	20	Extensive pigment cells, little nerve
	11	0.0 Li	3.5	1.0 Bi	20	Nerve

pigment cells induced by lithium. In experiment 8 the epithelium, which was not affected by lithium chloride, was induced to nerve by magnesium sulfate.

Using all the data in the experiments in Table IV we arrive at the following interpretation. After induction there is a short period during which the process is reversible. Once this primary induction is fixed, however, a strong neural inductor cannot reverse the process and pigment cells will differentiate after treatment with magnesium sulfate.

Another set of experiments testing the reversibility of induction of pigment cells is recorded in Table V. In all these experiments lithium chloride was first

used to induce pigment cells and then the aggregates were cultured continuously in a neural inductor. Experiments 1–4 show reversibility of induction as a result of culture in magnesium sulfate, while experiments 5 and 6 give the data for reversibility due to culture in sodium bicarbonate. As can be seen in experiment 6, a few pigment cells are able to differentiate in the presence of the neural inductor, sodium bicarbonate, and this fact reinforces the interpretation that the neural inductor reverses induction of, but does not interfere with the differentiation of, pigment cells.

Finally, we have a little data on the action of calcium chloride on reversibility of induction. Table VI shows that this compound in a concentration of 2.5 mg./ml., applied for two hours after induction of pigment cells by lithium chloride, changes pigment cells to slate gray epithelium, or changes slate gray epithelium to nerve. If, on the other hand, the aggregates are cultured continuously in a low

TABLE VI

Reversibility of induction of pigment cells Li: $LiCl$; Ca: $CaCl_2 \cdot 2\,H_2O$

Exp. no.	Stage no.	Li		Ca		No. of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11— 11 —	4.3 4.3	1.0 1.0	2.5 0.0	2.0 2.0	75 75	Nerve Slate gray epithelium, nerve
2	11 11	4.3 4.3	1.0 1.0	2.5 0.0	2.0 2.0	75 75	Slate gray epithelium, nerve Pigment cells, nerve
3	11 11	4.0 4.0	2.0 2.0	0.54 0.0	— —	75 75	Extensive pigment cells, nerve Few pigment cells, nerve
4	11 —	3.0	2.5	0.54	—	75	Pigment cells, nerve

concentration of calcium chloride after induction by lithium chloride, as in experiments 3 and 4, extensive pigment cell differentiation can occur.

Simultaneous action of two inductors

As a matter of empirical interest we wanted to see what sorts of induction would occur when two inductors were permitted to act at the same time. Since they do act synergistically in one sequence and antagonistically in the reverse sequence, it would be difficult to predict what would happen when they act simultaneously. In the design of the experiment two difficulties arose: (1) the choice of the relative concentration of the two inductors; and (2) the total ionic strength, which in combination is much higher than when applied singly in sequence. To some extent these problems can be met by using sodium bicarbonate as the second inductor, since it induces nerve cells in relatively low concentration. Experiments 1 and 2 in Table VII summarize the data we have on the action of lithium chloride and sodium bicarbonate in combination. While lithium chloride alone induces extensive pigment cells, in most cases when combined with sodium

bicarbonate, few or no pigment cells differentiate. Similar results are obtained with calcium chloride and magnesium sulfate in experiment 3, 5, 6, 7, 8, 9. On the other hand, experiments 4 and 10 would suggest that a more extensive set of experiments, involving different concentrations of the two inductors, might show a synergistic action. These two experiments involved lower concentrations of

TABLE VII

Simultaneous action of two inductors
Li: $LiCl$; *Bi*: $NaHCO_3$; *Ca*: $CaCl_2 \cdot 2\,H_2O$; *Mg*: $MgSO_4 \cdot 7\,H_2O$

Exp. no.	Stage no.	Treatment			Number of aggregates	Types of cellular differentiation
		Conc.	Hrs.	pH		
	11	2.0 Li + 1.5 Bi	2	9.0	25	Spreading nerve
	11	2.0 Li + 0.75 Bi	2	8.8	25	Spreading nerve
	11	2.0 Li	2	8.0	35	Nerve, pigment cells
	11	2.0 Li + 1.5 Bi	4	9.0	25	Nerve, few pigment cells
1	11	2.0 Li + 0.75 Bi	4	8.8	25	Nerve, few pigment cells
	11	2.0 Li	4	8.0	35	Extensive pigment cells, little nerve
	11	2.0 Li + 1.5 Bi	6	9.0	25	Nerve, few pigment cells
	11	2.0 Li + 0.75 Bi	6	8.8	25	Nerve, few pigment cells
	11	2.0 Li	6	8.0	35	Extensive pigment cells, little nerve
2	11	2.0 Li + 1.0 Bi	4	8.8	75	Spreading nerve
	11	2.0 Li + 1.0 Bi	7	8.8	75	Spreading nerve
	11	2.0 Li	4	8.0	35	Extensive pigment cells, nerve
3	11	2.0 Li + 2.0 Ca	3		60	Nerve, rare pigment cells
	11	2.0 Li	3		60	Extensive pigment cells, little nerve
4	11	0.5 Li + 1.5 Mg	7		25	Epithelium, few pigment cells, nerve
	11	0.5 Li	7		25	Ciliated masses, mucus
	11	1.5 Mg	7		25	Epithelium, ciliated masses
5	11	4.0 Li + 6.0 Mg	2		50	Unknown cell types
6	11	2.0 Li + 4.0 Mg	3		75	Spreading nerve, short nerve
7	11	2.0 Li + 3.0 Mg	2.0		40	Nerve, few pigment cells
	11	2.0 Li	2.0		35	Extensive pigment cells
	11	2.0 Li + 3.0 Mg	4.0		40	Nerve, few pigment cells
	11	2.0 Li	4.0		35	Extensive pigment cells
8	11	2.0 Li + 3.0 Mg	2.0		40	Nerve, slate gray epithelium
	11	2.0 Li	2.0		35	Nerve, pigment cells, slate gray epithelium
	11	2.0 Li + 3.0 Mg	3.0		40	Nerve, pigment cells
	11	2.0 Li	3.0		35	Extensive pigment cells, nerve
9	11	2.0 Li + 3.0 Mg	2.5		60	Nerve, slate gray epithelium
	11	2.0 Li	2.5		60	Nerve, some pigment cells
10	11	1.0 Li + 3.0 Mg	3.7		75	Spreading nerve, pigment cells
	11	1.0 Li	3.7		75	Radial nerve, rare pigment cells

lithium chloride combined with magnesium sulfate applied for a longer period of time. Note that while neither lithium chloride nor magnesium sulfate induced nerve or pigment cells, the combination induced some nerve cells and a few pigment cells. Clearly no final conclusions can be drawn from the data in Table VII.

Is the sequence of inductions a necessary one?

From a theoretical point of view it is important to know whether in the sequence of inductions the steps are so related that one depends upon another, or whether a specific inductor could induce one cell type without first inducing the cell type preceding. More specifically, is it possible to induce pigment cells without first inducing radial nerve and then spreading nerve? This question arose from speculation that the sequence of inductions might be almost entirely the result of a sequence in competence of the presumptive epidermis.

We first determined the change in competence with time of the presumptive epidermis to form pigment cells when treated with a specific concentration of lithium chloride for a specific time (3.0 mg./ml. for two hrs.). These results are

TABLE VIII

Sequential induction by lithium chloride in relation to competence

Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
	Conc.	Hrs.		
11+	3.0 Li	0.5	25	Epithelium, little nerve
11+	3.0 Li	1.0	25	Spreading nerve, few pigment cells
11+	3.0 Li	1.7	25	Extensive pigment cells, nerve
12−	3.0 Li	0.5	25	Epithelium, little nerve
12−	3.0 Li	1.0	25	Nerve, pigment cells
12−	3.0 Li	1.5	25	Extensive pigment cells, nerve

published in abstract form (Barth, 1964). The peak of competence was found to be between stages 11 plus and 12 minus. We then treated presumptive epidermis at these stages with lithium chloride for different lengths of time. The results are shown in Table VIII.

At both stages lithium chloride induces extensive pigment cells but as the duration of treatment is shortened nerve is induced instead of pigment cells. Thus, at the peak of competence for induction of pigment cells, lithium first induces nerve cells which then become further induced to pigment cells.

DISCUSSION

The process of induction in a sequence involves at least three basic phenomena. First, a cell type is determined and will reproduce itself during cell division. Second, the induction becomes irreversible, and thus the cell, having been induced, is inhibited in its capacity for differentiation into those cell types which precede it in the sequence. Third, the induction makes possible further induction, *i.e.*, the next step in the sequence of inductions. Thus, in the induction of

a nerve cell from the presumptive epidermis we need the activation of a self-duplicating system, which is best provided by DNA. We also need an inhibitor to prevent the induced cell from becoming an epidermal cell. And in addition, the induction of a nerve cell makes possible the further induction of a pigment cell.

In the normal course of events the presumptive epidermal cell undergoes cell division and differentiates. Thus, the DNAs for epidermal cell differentiation are active. When the cell is induced not only must the DNAs for nerve cells be activated, but those for epidermis must be inhibited. Finally, after the DNAs for nerve cells are activated, and only after this induction, we find that pigment cells may be induced. Thus, the induction of nerve cell must result in the competence for pigment cell induction.

The most direct approach to the problem outlined above is to assume that in the early determination of a cell by induction at least three DNAs are activated as a cistron. One DNA results in the synthesis of a specific protein which determines the differentiation of the cell. Another DNA results in the formation of a protein which masks the preceding DNAs in the sequence of inductions. A third DNA would direct the synthesis of a protein which would confer competence for the next step in the sequence. This latter competence might simply be a partial unmasking of the DNA in a cistron for the next cell type in the sequence so that an inductor might be able to complete the unmasking.

The action of inorganic ions as inductors

Of the known effects of lithium chloride on cells, the increase in viscosity of proteins (Ranzi and Citterio, 1957) is the most directly related to any possible action upon nucleoproteins. A change in structure of the histones by lithium chloride may possibly free the sites of action of the DNA and start the sequence of inductions. Nuclear histones, as regulators of gene activity, have been discussed recently by several investigators, including Busch *et al.* (1963), Bloch (1963), Horn (1962), and B. C. Moore (1963). Moore suggests that the genes are closely associated with histone and are prevented from synthesizing messenger RNAs before gastrulation. After gastrulation the association between histone and DNA changes and messenger RNAs are synthesized.

Using the above hypothesis, two mechanisms are needed for controlling the association between histone and DNA. During gastrulation there is a change in competence with time, which is independent of inductors. This is an intracellular control of gene action. Second, the inductors act on the competent cells and new genes must be activated. This is an extracellular control of gene action.

The first mechanism need not necessarily involve the actual removal of histone with the resultant activation of the genes, but rather make the histone susceptible to the action of an inductor. The repeated cell divisions during gastrulation, with the resultant exposure of the chromosomes to the action of cathepsins, may possibly remove most of the histone but leave enough to block one or more active sites on the DNA molecule. Then an inductor by uniting with histone may remove the last barrier to gene action.

Another possible way of reducing the histone is by repeated cell division. If DNA replicates faster than histone is synthesized, then the ratio of histone to DNA would be lowered. Extending this process beyond gastrulation, a time might

arrive when the genes for the differentiation of epidermis were activated by removal of histone. No extracellular inductor is necessary for the determination of ciliated cells of the epidermis.

Quite clearly the main difficulty in the hypothesis of the control of gene action by histones is lack of evidence for the specificity necessary to activate one set of genes in one group of cells and another set in a second group of cells. Obviously some histones would need to remain in association with DNA while others would be removed. In the case of sequential induction the histone associated with neural genes would need to be more susceptible to the action of inductors than the histone combined with pigment cell genes. The concept of sequential induction might be of help in reducing the numbers of histones to the numbers of sequential inductions. Thus, perhaps one kind of histone is associated with those genes involved in ectodermal differentiation, another in mesodermal differentiation and a third in endodermal differentiation. In any one sequence of inductions the amount of histone might possibly determine the susceptibility of any one set of genes to the action of an inductor.

Relation of sequential induction to the organizer

In the case of the organizer phenomenon we have a situation by which chordamesoderm induces the presumptive neural plate to form the definitive neural plate, and at the same time becomes definitive notochord and mesoderm. The median portion of the neural plate differentiates into motor neurons, while the lateral regions form neural crests which differentiate into sensory neurons, sympathetic neurons, pigment cells, adrenal medulla and other cell types. The presumptive chordamesoderm has competence for differentiation into neural plate and the presumptive neural plate has competence for differentiation into chordamesoderm. Thus, one basic question arises: Why doesn't the presumptive chordamesoderm differentiate into neural plate since it has both the competence and the inductor for neural plate? Next, we have to account for the induction of forebrain by the prechordal plate and spinal cord by the chordamesoderm. In addition, we would like to have an explanation for the medio-lateral differences in the neural plate, since these arise shortly after the time of induction (Corner, personal communication).

One basic assumption will be made. The living organizer produces an inductor which induces different cell types at different concentrations. The highest concentration of the inductor will then be in the chordamesoderm itself and will induce these cells to become notochord and mesoderm. As gastrulation occurs, the next highest concentration of the inductor will be in the posterior presumptive neural plate, because this region is in contact with the inductor for the longest period of time, and also the chordamesoderm has greater mass per unit area in this region. The anterior presumptive neural plate will have the lowest concentration of the inductor for two reasons. First, it is in contact with the prechordal plate for only a short time before competence is lost, and second, because the prechordal plate is very thin and the concentration of the inductor must be correspondingly low. Mangold (1933) commented on the very weak inductive capacity of the prechordal plate.

Within the chordamesoderm during gastrulation a situation develops whereby

the notochord is a thin strip of tissue applied to the median region of the presumptive neural plate, while the mesoderm becomes concentrated in a much thicker layer under the lateral regions of the neural plate. Thus, the concentration of the inductor will be higher in the lateral regions as compared with the median region.

Therefore, we visualize a situation by which a single inductor, at a concentration C_n induces chordamesoderm; at a concentration of C_{n-1} induces neural crest; at a lesser concentration, C_{n-2}, spinal cord; at C_{n-3}, forebrain. What evidence do we have to support this hypothesis?

With regard to induction of forebrain, most of the evidence supports a weak inductor. (1) In *Ambystoma maculatum* no external inductor at all is necessary for induction of forebrain (Barth, 1941; Holtfreter, 1944). (2) The spinocaudal inductor, when partly denatured, induces forebrain (Yamada, 1958). (3) The mesodermal inductor, when heated, loses its inductive properties in the order of spinocaudal, hindbrain, forebrain, to no induction (Yamada, 1958). (4) Mangold (1933) tested the anterior region of the roof of the archenteron in the blastocoel of an early gastrula and found it to have weak inductive capacity as compared with more posterior regions.

The induction of the medio-lateral regionality in the neural plate can be imitated to some extent by lithium chloride. With a low concentration of, or short exposure to, lithium chloride, the presumptive epidermis forms motor neurons, as evidenced by their action to stimulate muscle to contract (Barth and Barth, 1959). Higher concentrations of lithium chloride or longer exposure time results in neural crest, as evidenced by pigment cell differentiation and a type of nerve which is similar to sympathetic nerve.

Finally, have we any single inductor which actually does induce mesoderm, notochord, and neural tissue? Masui (1960) has obtained excellent induction of notochord, muscle, pronephros, nerve, pigment cells and mesenchyme by the use of lithium chloride. In our own experiments we were not so successful as Masui, but we did obtain at least two types of nerve, pigment cells, mesenchyme, but only sporadically notochord and muscle. Finally, it is clear that one substance is able to induce differentiation of many cell types, and we believe that this constitutes good evidence for a single inductor in the organizer region of the gastrula.

A number of objections may be made against the hypothesis of a single inductor present in the organizer region and acting by inducing various cell types at various concentrations. Among these objections we will consider two. First, how can we reconcile the fact that one half of the organizer region will induce a bilaterally symmetrical neural plate? The differences in concentration of a single inductor would be expected to be radically altered after bisection of the organizer, and an asymmetrical neural plate would result.

Second, the fact that a non-organizer region containing no inductor may be placed in the organizer region with no change in the neural plate induced requires an explanation. Small transplants of presumptive epidermis placed in the organizer region regulate, undergo gastrulation and become inductors. How can the differences in concentration of the inductor required by the hypothesis be maintained under these circumstances?

The two criticisms chosen above may be leveled at most hypotheses of organizer action and are not particular criticisms of the single inductor hypothesis. Indeed,

it would appear to be even more difficult to interpret the experiments on division of the organizer and substitution of the organizer if the hypothesis of a mosaic of inductors is offered.

Summary

1. A sequence of inductions has been obtained with a single inductor applied to the presumptive epidermis in various concentrations and for varying lengths of time.

2. A neural inductor, such as magnesium sulfate or calcium chloride, will induce a part of the sequence such that a subminimal exposure to lithium chloride then is able to induce pigment cells.

3. After lithium chloride is used to induce pigment cells, a strong neural inductor such as sodium bicarbonate, magnesium sulfate or calcium chloride applied immediately after lithium treatment may reverse the induction and nerve cell differentiation will result.

4. The sequence of inductions is a necessary one, as evidenced by the fact that at the peak of competence for pigment cell induction, lithium chloride first induces nerve cells and then pigment cells.

LITERATURE CITED

Barth, L. G., 1941. Neural differentiation without organizer. *J. Exp. Zool.*, **87**: 371–384.

Barth, Lucena Jaeger, 1964. Control of cellular differentiation by inorganic ions in relation to competence. *Amer. Zool.*, **4**: 317.

Barth, L. G., and L. J. Barth, 1959. Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. Exp. Morphol.*, **7**: 210–222.

Barth, L. G., and L. J. Barth, 1962. Further investigations of the differentiation *in vitro* of presumptive epidermis cells of the *Rana pipiens* gastrula. *J. Morphol.*, **110**: 347–373.

Barth, L. G., and L. J. Barth, 1963. The relation between intensity of inductor and type of cellular differentiation of *Rana pipiens* presumptive epidermis. *Biol. Bull.*, **124**: 125–140.

Bloch, D. P., 1963. Genetic implications of histone behavior. *J. Cell. Comp. Physiol.*, **62**: 87–94.

Busch, H., W. J. Steele, L. S. Hnilica, C. W. Taylor, and H. Mavioglu, 1963. Biochemistry of histones and the cell cycle. *J. Cell. Comp. Physiol.*, **62**: 95–110.

Holtfreter, J., 1944. Neural differentiation of ectoderm through exposure to saline solution. *J. Exp. Zool.*, **95**: 307–340.

Horn, E. C., 1962. Extranuclear histone in the amphibian oocyte. *Proc. Nat. Acad. Sci.*, **48**: 257–265.

Mangold, O., 1933. Über die Induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwiss.*, **43**: 761–766.

Masui, Y., 1960. Alteration of the differentiation of gastrula ectoderm under influence of lithium chloride. *Mem. Kōnan Univ., Sci. Ser., No. 4, Art. 17*, 79–102.

Moore, Betty C., 1963. Histones and differentiation. *Proc. Nat. Acad. Sci.*, **50**: 1018–1026.

Ranzi, S., and P. Citterio, 1957. Proteine e determinazione embrionale nella *Rana*, nel riccio di mare e negli incroci dei rospi. *Acta Embryol. et Morph. Exp.*, **1**: 78–98.

Shumway, W., 1940. Stages in the normal development of *Rana pipiens*. *Anat. Rec.*, **78**: 139–147.

Yamada, T, 1958. Embryonic induction. *In:* The Chemical Basis of Development, pp. 217–238. Ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md.