Chemical Messengers in Development: A Hypothesis

Differentiation of embryonic cells may be determined by their content of inorganic ions and cyclic nucleotides.

Daniel McMahon

Developmental induction and morphogenetic fields work together during development to shape the eggs and the embryos of many species of animals into adults. They allow a cell to determine the direction of its further differentiation on the basis of information about its position in the embryo. This information is presumably transduced into one or more internal chemical signals that regulate the metabolism of the cell and the differential expression of its genes, thereby regulating differentiation, multiplication, or movement of the cell.

The cell appears able to make only a small number of choices based upon information about its position. Consideration of the number of alternative developmental fates normally available to a cell and of the range of possible "transdeterminations" of the fate of developing cells has led to the suggestion that very few, perhaps only two, choices of developmental path are available to the cell (*1, 2*) at a given point in development although in some cases more may be available (*3*). Additional choices are made during development, and the cell and its progeny become committed to an increasingly circumscribed fate (*4*).

The organization of morphogenetic fields differs from that of induction in at least one important respect. All cells in a morphogenetic field are equivalent in their interactions; they both generate and respond to the positional information (*1, 4*). However, there are two distinctly different groups of cells that participate in every induction. Inducing cells or chemicals produced by them act on inducible cells, and they or an equivalent source of inducing chemicals are necessary for each induction (*4, 5*). Therefore, the process of induction has been more amenable to biochemical investigation than the process of morphogenetic field organization.

Some inductions require direct contact between the inducing and inducible cells (*5, 6*) while others are mediated by diffusible substances (*5, 7, 8*) that can diffuse only a short distance (60 to 70 micrometers) (*7, 8*). Although the induced cells may be able to transmit an inductive stimulus (*9*), they do not acquire the structural or functional properties of the inducing cells (*5*). Cells that produce collagen (*10, 11*) and nervous tissue (*7, 12*) are often inducing tissues. Evidence discussed below suggests that collagen and neurotransmitters may be directly involved in the biochemistry of induction.

Inducers

Cells and macromolecules. While natural inductions have specific requirements for inducing and inducible cells, many experiments have shown that both common and exotic reagents can induce cell differentiation or modify entire embryonic axes when added to a developing organism. For example, heterologous adult tissues, including heat-killed HeLa cells and alcohol-fixed liver or kidney cells, induce specific cell and tissue differentiation when implanted into amphibian embryos (*5, 13*).

Cell fractions can also induce differentiation. Microsomes induce the formation of rear brain and spinal structures in amphibian embryos (*14*). Since ribosomelike particles have been observed in the spaces separating inducing from inducible tissues and gradients of RNA content have been found in the cells of some embryos, the possibility that RNA might mediate some inductions has been extensively studied. Although many investigators have attempted to detect the transfer of RNA from inducing to inducible tissue or to produce inductions with pure RNA (*15*), the results of the reported experiments remain equivocal (*16*).

Investigations of the roles of proteins in induction have been more rewarding. Tiedemann *et al.* (*17*) partially purified inducer proteins, but the mechanism of action is unknown. Levine *et al.* have shown that the site of action of a protein inducer of pancreatic differentiation appears to be the plasma membrane of the inducible cells (*18*). Collagen has been implicated in induction. A protein resembling collagen in composition is transferred to inducible tissues during some inductions (*10*), and some inductions can be prevented by treatment of the developing cells with collagenase (*11, 19*). A meshwork of collagen seems to determine the sites of scale and feather formation. In both fish (*20*) and chickens (*11, 20*), cells accumulate at the intersections of collagen fibers and become the germs for scales or feathers. In the chicken embryo, treatment with hydrocortisone abolishes both the collagen mesh and feather induction, and a mutant which lacks feathers also lacks the collagen mesh (*20*). The ability of collagen to stimulate cell growth (*21*) and a requirement for collagen for differentiation of myoblasts in vitro (*22*) provide more direct evidence that collagen may have some role as a regulator of development. Its polarity, insolubility, and susceptibility to secondary modification (*23*) could be useful properties for a molecule guiding some aspects of development as both Trelstad and Gross (*24*) have suggested.

Ions. At the other end of the scale of chemical complexity, simple cations induce cellular differentiation and alter developmental axes (*25*). It has been argued (*26*) that the effects of cations on the differentiation of amphibian cells may result from cell damage, but this appears to be unlikely (*27*). Great increase in the flux of ions across the plasma membrane occurs naturally at the time of induction, as might be expected if ions were involved in natural inductions. Some of the increase in flux may result from the release of bound ions in the cell (*28*).

The author is an assistant professor in the Division of Biology, California Institute of Technology, Pasadena 91109.

Barth and Barth (*27*) have examined the effects of cations on the very early development of the frog and have shown that the cations Li^+, Na^+, or Ca^{2+} induce differentiation of neurons from presumptive epidermis. These neurons can be further induced to differentiate into pigment cells by later treatment with the same ions. Three types of neurons or pigment cells are produced by sequential inductions. Several lines of evidence suggest that these ions are triggering the natural biochemical processes which produce induction. The ectodermal cells are competent for these inductions only at the stages of development at which the inductions normally occur in the embryo. The result of treatment of the cells with the ionic inducer changes similarly with time, whether the cells are left in the embryo or cultured in vitro. Ectodermal cells cultured in vitro must be treated with an ion which can trigger them to become neurons before they are competent to be induced to become pigment cells although the same inducer (such as Li^+) can effect both inductions. Changes in ionic flux normally accompany the inductions of nervous tissue in vivo. These changes do not occur in an interspecific hybrid which does not gastrulate normally and in which, therefore, the ectoderm is not subjected to the natural inducer. The neural plate and its derivative cells are not induced, and the normal changes in permeability to ions do not occur. Cells from these abnormal gastrulas can be induced to form neurons and pigment cells by treating them with the same ions which induce neural differentiation in the cells of a normal embryo. This shows that the hybrid cells contain the information to make neurons and pigment cells and can express this information if triggered. These experiments further suggest that the ionic changes which accompany the induction in vivo result from the process of induction and are sufficient for the induction of neurons and pigment cells.

The multiplicity of inducers and the apparent lack of an underlying chemical mechanism for their action make the phenomenon of induction difficult to understand and provide an incentive to further the belief that the process is an artifact. A simple explanation underlies the apparent complexity. Developmental processes are normal physiological functions and are guided by the same molecules that are used to regulate the physiological state of the adult.

Biochemical Messengers in Development

The following biochemical mechanism is proposed for transduction of positional information in development:

1) The developmental path of a cell is determined by the temporal sequences of changes in the concentrations of cyclic nucleotides and inorganic ions in the developing cell. The intracellular inorganic ions and cyclic nucleotides combine to regulate metabolism and expression of genes in the developing cell. They may also regulate one another's concentration within the cell.

2) First, inducers bind to receptors on the plasma membrane. The spectrum of receptors on its plasma membrane and the cell's repertoire of possible responses to changes in the concentrations of cyclic nucleotides and inorganic ions result from the cell's previous differentiation.

Second, inducers are the molecules that regulate cyclic nucleotide and ion concentrations in the cells of the adult. They include neurotransmitters, polypeptide hormones, collagen, lectinlike proteins, and prostaglandins. Inductions mediated by cell contact are produced by similar molecules attached to the surface of other developing cells.

My perspective differs from that of others who have directed many recent studies of induction. They (*15*) have apparently been motivated by the assumption that only a substance with a high content of information could guide a cell through its differentiation, and have looked for the transfer of RNA or similar molecules from the inducing to the inducible cells. My model is based on the assumption that a major part of the information that guides the cell is contained within the inducible cell itself, and in this respect the model returns to the ideas of Holtfreter (*29*) and others (*27, 30*).

The suggestion that neurotransmitters are involved in developmental regulation has already been made (*12, 31–33*), but no biochemical mechanism for their action has been offered. In one case this hypothesis has been rejected because some inhibitors of neurotransmitter action do not block development (*34*). I believe that those effects of neurotransmitters on the cell which alter the cellular concentrations of cyclic nucleotides in the cell are very important so that inhibitors which do not block these changes [including those used in that study (*34*)] may have little or no effect on development.

Cyclic Nucleotides and Their Effects

Cell biology of cyclic nucleotides. The concentrations of cyclic adenosine monophosphate (AMP) and cyclic guanosine monophosphate (GMP) are physiologically regulated in the cell, and changes in their concentration have major biological effects. Each is made and degraded in an analogous process. For example, adenosine triphosphate (ATP) is converted to cyclic AMP plus pyrophosphate by the enzyme adenylate cyclase and is degraded to 5′-AMP by phosphodiesterases. Most eukaryotic cells have a phosphodiesterase specific for cyclic AMP and one specific for cyclic GMP. The cellular concentration of cyclic nucleotide can be physiologically or experimentally manipulated either by regulating its rate of synthesis or its rate of degradation. In eukaryotes, although adenylate cyclase is bound to cellular membranes, guanylate cyclase and the phosphodiesterases may be either membrane-bound or soluble, depending on cell type (*35*).

The work of Sutherland and Rall (*36*) on the regulation of carbohydrate metabolism in the liver, which first led to the concept of cyclic AMP as the second messenger of hormones has resulted in a phenomenal explosion of knowledge about the regulation and the effects of the cyclic nucleotides. Some of the physiological agents that regulate the concentrations of cyclic AMP or cyclic GMP are listed in Table 1. With the exception of the prostaglandins, one or more of the compounds in each group has been shown to affect development. Each cell type has a pattern of sensitivity to these agents although some compounds stimulate cyclic AMP synthesis in many kinds of organisms. The neurotransmitters serotonin and epinephrine activate the adenylate cyclase of mammalian cells and of the protozoans *Tetrahymena pyriformis* and *Euglena gracilis* (*37*). The mammalian hormone glucagon regulates glycogenolysis in the liver and in the fungus *Neurospora crassa* (*38*) by stimulating activity of adenylate cyclase.

The processes that the cyclic nucleotides regulate completely overlap the characteristic processes of development and differentiation. For example, cyclic AMP regulates the morphology (*39*), motility (*40*), and pigmentation of cells (*41*). In addition, exogenously applied cyclic AMP inhibits cell division in some cultured cells, and the concentrations of cyclic AMP in cells

Table 1. Compounds which physiologically increase cyclic nucleotide concentrations.

Compounds	References
Compounds which increase cyclic AMP	
Polypeptide hormones (glucagon, adrenocorticotrophic hormone, secretin, pancreozymin, thyroid stimulating hormone, vasopressin, parathyroid hormone, melanocyte stimulating hormone, and others)	(*38, 125–130*)
Neurotransmitters (norepinephrine, epinephrine, dopamine, serotonin, histamine)	(*37, 125, 130–133*)
Prostaglandins E_1, E_2, and others	(*126, 128, 134*)
Thyroxine	(*97*)
Ecdysone	(*135*)
Compounds which increase cyclic GMP	
Polypeptide hormones (insulin, secretin, ocytocin)	(*73, 129*)
Neurotransmitters (acetylcholine, serotonin, norepinephrine, histamine)	(*73, 126, 127, 136–138*)
Prostaglandin $F_{2\alpha}$	(*73*)
Collagen	(*73*)
Lectins (concanavalin A, phytohemagglutinin)	(*56, 58*)

increase as multiplication ceases in some cultured cell lines (*42*), but added cyclic AMP can stimulate proliferation in others (*43*). This indicates that the spectrum of effects of cyclic AMP is a function of cell type. Thus cyclic AMP enhances pigment production in cells derived from melanocytes. Depending on the kind of cell treated with cyclic AMP, it induces the morphology of a fibroblast, astrocyte, or neuron (*39*); and, although it inhibits the mobility of fibroblasts, it stimulates the motility of sperm (*40*).

Cyclic AMP produces considerable effects on cellular organelles. It enhances the stability and stimulates the assembly of microtubules (*39*). It also affects a number of properties of the plasma membrane, including cellular permeability (an effect opposed by cyclic GMP), adhesiveness, and composition (*44, 45*). Cyclic AMP is also involved in regulating gene expression. Increase in cellular cyclic AMP produced by hormonal treatment or by treatment of cells with dibutyryl cyclic AMP stimulates both the synthesis of enzymes and other proteins in vivo (*39, 46–48*), the synthesis and modification of nuclear proteins (*46, 49, 50*), the puffing of *Drosophila* chromosomes (*51*), and the synthesis of RNA in vivo (*52*). Similar results have been demonstrated in vitro where cyclic AMP regulates the synthesis of proteins and RNA (*53*) and stimulates the phosphorylation of ribosomal (*45, 54*) and chromosomal proteins (*50*).

Cyclic GMP has not received nearly as much experimental attention. Nevertheless, at concentrations of 5 picomoles per liter it triggers DNA synthesis in stem cells of the bone marrow and stimulates cell division in lymphocytes (*55*). Stimuli which increase the concentration of cyclic GMP in the lymphocyte produce blast transformation (*56*) causing the transformed cells to grow in size, synthesize RNA at a greater rate, and multiply. Cyclic GMP also stimulates protein and RNA (*53, 57*) synthesis in vitro. In many systems it appears to antagonize the physiological effects of cyclic AMP (*35, 44, 58*).

Biochemical Mechanism of Action

Although the biochemical mechanisms through which cyclic nucleotides exert their effects are not yet completely resolved, two mechanisms of their action have been uncovered. The first has only been described in a prokaryote. In *Escherichia coli*, cyclic AMP regulates the expression of some genes by activating a cyclic AMP binding protein that stimulates the transcription of a defined subset of genes by directly interacting with the transcriptional complex (*59*). Cyclic GMP normally antagonizes this effect of cyclic AMP, but the specificity of the cyclic AMP binding protein can be reversed by mutation so that it is activated by cyclic GMP (*60*).

Cyclic AMP–dependent protein kinases occur in both prokaryotes and eukaryotes. When activated by cyclic AMP, they transfer a phosphate group from ATP to proteins including enzymes and histones (*50, 61–65*). Enzymes may be activated or inactivated by phosphorylation. Cyclic AMP–activated kinases occur in at least nine phyla of the animal kingdom (*62*). Their metabolic role and wide distribution have led Kuo and Greengard (*61*) to suggest that they are the primary mechanism of exerting the effects of cyclic AMP. It is too early to evaluate this hypothesis, but it is clear that hormone-produced increase in cyclic AMP in living cells activates these enzymes (*66*), and that they produce at least some of the metabolic effects of cyclic AMP in vitro (*63, 67, 68*). Either cyclic AMP binding proteins or protein kinases provide attractive models for the regulation of gene expression by cyclic AMP in development.

Changes in the cellular concentration of cyclic AMP might appear to provide only two choices for the developing cell. However, they could produce several effects on the cell depending on the magnitude of the change in cyclic AMP concentration and on the ionic conditions in the cell. There are two to seven different cyclic AMP–activated protein kinases in many mammalian tissues (*64, 65, 68*), and, in some cases, each has been shown to be independently regulated. The effects of cyclic AMP could be altered in other ways. The substrate specificity of cyclic nucleotide–dependent protein kinases often differs and can be modified by a protein factor (*69*). Protein phosphatases activated by cyclic AMP can reverse the effects of protein kinases by dephosphorylating proteins (*44, 70*). Changes of cyclic AMP concentration of different magnitude could produce a variety of effects in the cell, depending on the spectrum of kinases and phosphatases in the cell and also on the relative sensitivity of these enzymes to cellular conditions. A model case, in this respect, is the regulation of glycogen phosphorylase kinase activity by both cyclic AMP and Ca^{2+} ion (*71*).

The biochemical mechanism of the effects of cyclic GMP has not been investigated as intensively as that of cyclic AMP. Nevertheless, cyclic GMP–dependent protein kinases occur in the arthropods (*69*) as well as in the pancreas and the cerebellum of the rat (*72*). The interactions of changes in cyclic GMP concentration within the cell with changes in cyclic AMP concentration have received very little attention from biochemists, but physiological studies indicate that in the interaction of cyclic AMP and cyclic GMP concentration is important. This idea has been developed by Goldberg in his yin-yang theory of cyclic nucleotide action (*73*) [and see also (*74*)].

Inorganic Ions and Cyclic Nucleotides

The effects and metabolism of cyclic AMP are intricately interwoven with the ionic conditions within and outside the cell. Cyclic AMP modifies the permeability of the cell to inorganic ions. Inorganic ions are necessary for the generation and destruction of cyclic AMP and for the action of cyclic AMP–dependent protein kinases and phosphatases (see Table 2). They may also be required for processes that occur subsequent to the regulation of cyclic nucleotide production and subsequent to their action (*75*). Treatment of developing cells with high concentrations of cations could affect the cyclic nucleotide–mediated system. Apparently cyclic GMP and inorganic cations are similarly coupled within the cell, but data on this subject are very scanty. The following discussion focuses on the relations between cyclic AMP and inorganic cations.

The effects of cyclic AMP on permeability have been studied most thoroughly in secretory and nervous tissue. Changes in permeability to or transport of K^+, Ca^{2+}, or Cl^- result from hormonally produced changes in cyclic AMP concentration or from topical application of cyclic AMP or its derivatives (*76*). The effects of cyclic AMP on ionic permeability vary with cell type. Besides changing the cellular permeability, cyclic AMP could change the intracellular concentrations of cations by liberating them from a sequestered intracellular pool. Cyclic AMP does release Ca^{2+} from such pools (*75*). The effects of cyclic AMP on the intracellular distribution of other cations have not yet been subjected to similar scrutiny.

Ions and cyclic AMP interact in several significant developmental processes. Low concentrations of Ca^{2+} are required for stimulatory effects of cyclic AMP on cell division (*43*). Uptake of K^+ mediated by the Na^+, K^+–adenosine triphosphate is required for blast transformation of lymphocytes (*76a*). The aggregation of the amoeba *Dictyostelium discoideum* depends on both the production of cyclic AMP by the amoebas and low (10^{-6} to $10^{-4}M$) concentrations of extracellular Ca^{2+} (*77*). Even though the amoebas produce cyclic AMP in the absence of Ca^{2+}, they cannot respond to it.

Examination of the data on the effects of some cations on the enzymes closely associated with cyclic AMP in the cell (Table 2) indicates that most of these ions when applied extracellularly might either promote or antagonize the effects of cyclic AMP, depending on the intracellular concentration of the ion and on the relative rates of enzymatic reactions. Two ions may have unidirectional effects on this system. Suitable concentrations of Li^+ and Ca^{2+} should inhibit the generation and actions of cyclic AMP. Particularly, Li^+ inhibits the hormone-stimulated fraction of adenylate cyclase activity. Because of its site of action, Li^+ could exert its effects without entering the cell. Since the effects of Li^+ are particularly evident on the adenylate cyclase of nerve cells, it may be possible to suggest an interpretation (unique to the model offered in this article) of the work of Barth and Barth (*27*) on the induction of the differentiation of neurons and pigment cells by ions—namely, that Li^+ and Ca^{2+} channel ectodermal cells into the path of differentiation which leads to neurons and pigment cells by lowering the cellular concentration of cyclic AMP or by reducing its effects.

Multiple Rounds of Induction

The experiments on the induction of nervous tissue by cations demonstrate that a substance of low information content can induce specific cell differentiation that is a function of the age of the cells and their previous inductions. Any biochemical mechanism for the specification of positional information in development must account for a series of sequential specifications of position (and the commitment to a specific pathway of development which results) that seems to occur over and over again in the embryo. Generation of a series of sequential inductions require (i) that developing cells become sensitive to new inducers or transduce the previous signal into a new internal messenger or (ii) that developing cells generate a new set of responses to changes in the same internal signal. For the model described in this article, which specifies that there is a very limited set of internal messengers, both responses may be necessary for multiple rounds of sequential induction.

This model is able to generate new responses to cyclic nucleotides as a

Table 2. The effects of inorganic cations on the metabolism and action of cyclic AMP.

Cation	Effects
Li^+	Lithium ion inhibits adenylate cyclase (*35, 139, 140*). The adenylate cyclase of brain is particularly sensitive to lithium inhibition (*139, 140*) but adenylate cyclase activity of many tissues (particularly the hormone-stimulated activity) is inhibited by lithium (*35, 139, 140*). Lithium ion (5 m*M*) can inhibit adenylate cyclase by 17 percent while 25 m*M* may inhibit 39 to 73 percent (*139, 140*). Lithium ion may also inhibit the effects of cyclic AMP (*140*).
Na^+, K^+	Sodium and potassium ions have slight effects on adenylate cyclase (*140*). A high concentration of either inhibits the ability of protein kinases to phosphorylate their substrates (*65, 67*). For example, 0.1*M* NaCl increases the Michaelis constant for casein by six times (*65*). However, a similar concentration may stimulate the phosphorylation of histones (*65*).
Ca^{2+}	The adenylate cyclase from brain (*35, 131, 141*) and at least one protein kinase (*63*) seem to require a very small amount of bound Ca^{2+} for activity. However, low concentrations ($\geq 10^{-4}M$) of Ca^{2+} inhibit adenylate cyclase (*35, 131, 141*) and protein kinases (*62, 67, 69, 142*) from brain and other tissues and may even cause cyclic AMP to inhibit cyclic AMP–dependent protein kinase (*62*). Cyclic AMP phosphodiesterase is also inhibited by Ca^{2+} ($> 10^{-4}M$) particularly in the absence of Mg^{2+} although smaller amounts of Ca^{2+} than $10^{-4}M$ may stimulate it (*143*). Calcium also inhibits the activity of phosphoprotein phosphatase (*68, 70*).
Mg^{2+}	Mg^{2+} or Mn^{2+} ions are required for the activity of adenylate cyclase (*35, 125, 144*), phosphodiesterase (*143*), and protein kinases (*62, 63, 65, 67, 68, 142*). The concentration of Mg^{2+} also affects the modulation of protein kinase activity by the cyclic AMP–dependent protein kinase modulator (*69*). Mg^{2+} is not required for the activity of at least one phosphoprotein phosphatase (*70*).
Mn^{2+}	Produces maximum activity of adenylate cyclase (*125, 144, 145*) and may stimulate or inhibit phosphodiesterase (*143*). Protein kinases vary in their response to Mn^{2+}. It may be as effective a cofactor as Mg^{2+} (*62*) or ineffective (*65, 67*), but in most cases it gives an enzyme activity intermediate between zero and maximum obtainable (*61, 142*). Mn^{2+} stimulates the activity of brain protein phosphatase (*71*).
Zn^{2+}	Zn^{2+} can inhibit adenylate cyclase (*144*), phosphodiesterase (*143*), and protein phosphatase (*71*). It does not support the activity of protein kinases (*65, 67, 142*) and does not appear to inhibit them.

result of a previous round of cyclic nucleotide stimulation. For example, new effectors of cyclic nucleotide action, such as protein kinases or phosphatases, could be synthesized, or new substrates for the effectors, such as chromosomal proteins or enzymes, might be made after each round of induction. Only two investigations bear on these possibilities: Rensing and Hardeland (*51*) have shown that dibutyryl cyclic AMP induces or represses chromosomal puff formation in larvae of *Drosophila melanogaster* at specific stages of larval development and that different puffs are induced by cyclic AMP at different stages of development. Since puffs in *Drosophila* are areas of enhanced transcription of DNA and are thought to be single functional units, this provides additional support for my suggestion that the effect of cyclic AMP on the regulation of gene expression changes during development. The developing mouse mammary gland is the second system which suggests that the effects of cyclic AMP can be altered as a result of previous stimuli which have influenced cyclic nucleotide metabolism. In this system prolactin can induce the regulatory subunit of the cyclic AMP–activated protein kinase, whereas the combined effects of insulin (Table 1) and prolactin are necessary to induce the catalytic subunit of the enzyme (*45, 78*).

Developing cells could become sensitive to a new set of positional signals by generating new receptors in response to induction, by modifying the internal signal produced in response to the inducer, or by modifying their environment (for example, by destroying the inducing molecules or by moving). Each of these is feasible in terms of this model. The sensitivity of frog erythrocytes (*79*), mammalian pineal gland, brain, and liver (*80*) to stimuli that regulate their concentration of cyclic AMP changes during development. In the lymphocyte, stimuli that trigger a large change in cyclic GMP concentration cause the appearance of surface insulin receptors on the cell while two other types of receptors maintain the same or decrease their density (*81*). The same stimulus (that is, many neurotransmitters) is able to regulate either cyclic AMP or cyclic GMP concentrations in vivo in different cell types (Table 1). Cells can also modify potential inducing molecules in their environment in response to changes in concentration of a cyclic nucleotide. Cyclic AMP activates, both in vivo and in vitro, collagenase in the tail of the tadpole (*82*). The activation of collagenase by cyclic AMP provides a model for a system where alterations of the content of cyclic GMP in collagen-sensitive cells could result from a previous increase of cyclic AMP concentration in the same cells or in neighboring cells (Table 1). Extensive tissue remodeling occurs in conjunction with the removal of collagen, as described above. It would be interesting to know whether the concentration of cyclic GMP changes in the cells of the tail during removal of the collagen but before the remodeling of tissue begins.

Inductions Mediated by Cell Contact or Insoluble Extracellular Materials

Cell contact has been shown to be important for the biochemical differentiation of cells from a variety of organisms and for a number of different kinds of cells, including chondrocytes and myoblasts (*83*). I have suggested a mechanism for the communication of positional information in morphogenetic fields which can also explain inductions mediated by cell contact and by insoluble extracellular materials and perhaps even those bizarre inductions produced by killed heterologous adult tissues. In this model, positional information in the embryo is transmitted by cell contacts that also regulate the intracellular concentrations of molecules like cyclic AMP or cyclic GMP. Two of the predictions of this model have already been verified in *Dictyostelium discoideum* (*84*). Molecules able to activate these contacts (as hormones do) but which do not respond to contact themselves would produce an induction that is transmitted by the induced cells.

Chemical Teratogenesis of the Chicken Embryo

Effects of insulin and pyridine nucleotides on development. Landauer and colleagues (*85*) have shown that some chemicals produce specific developmental (*86*) defects when injected into yolk of the developing chicken embryo. The frequency and severity of the defects depend critically upon the stage of development at which the injection is made. Injection into the embryo of 0.1 to 0.5 unit of insulin per milliliter of yolk affects cellular differentiation and morphogenesis. It shortens the long axis of the vertebral column (the vertebrae of the tail do not develop), the beak, and the limbs (*85, 86*), and prevents closure of the neural tube. The treatment produces abnormally short leg bones but this is not the result of an extensive physiological change, such as hypoglycemia, and can be produced on bones cultured with insulin in vitro (*85, 86*). Similar abnormalities occur in humans. The frequency of children with congenital malformations born to diabetic mothers receiving insulin therapy is much greater than normal (*88*), including an increased frequency of the absence of the lower half of the vertebral column.

Another teratogen, 3-acetylpyridine ($\sim 2 \times 10^{-4}M$ to $6 \times 10^{-4}M$) has little effect on the bones but produces severe hypoplasia of the skeletal muscles of the leg (*85, 89*), but this effect of 3-acetylpyridine can be completely prevented by insulin (~ 0.4 unit/ml) (*85*) or nicotinic acid ($\sim 1 \times 10^{-4}M$). In vitro, 3-acetylpyridine ($\sim 0.01M$) partially relieves the dependence of chondrogenesis on cell density (*90*), stimulates the extent of chondrogenesis (*90*) and the production of enzymes associated with the synthesis of cartilage (*91*), and may trigger uncommitted mesodermal cells into differentiation as chondrocytes (*90*). It inhibits the proliferation and differentiation of myoblasts (precursor cells of skeletal muscle) in vitro (*90, 92*), in agreement with its effects in vivo. Nicotinic acid prevents the effects of 3-acetylpyridine on both chondrocytes and myoblasts.

A proposed involvement of cyclic nucleotides. The effects of insulin and 3-acetylpyridine and related compounds on development that have been suggested to result from changes in pyridine nucleotide (*85–87, 90*) or carbohydrate (*93*) metabolism in the affected cells can be interpreted in another way. Nicotinic acid (100 μM) inhibits the production of cyclic AMP in many tissues by inhibiting adenylate cyclase at the concentrations of nicotinate which are effective in these experiments (*94*). At higher concentrations ($1 \times 10^{-4}M$ to $2.5 \times 10^{-3}M$) it prevents the effects of exogenous dibutyryl cyclic AMP (*95*). The effect of 3-acetylpyridine on the cyclic AMP system is opposite that of nicotinic acid since it inhibits cyclic AMP phosphodiesterase and increases cyclic AMP (*96*). Therefore, I assume that nicotinic acid decreases the concentration

of cyclic AMP in the mesodermal cells of the leg while 3-acetylpyridine increases it. Insulin produces large increases in cyclic GMP in all cells and tissues studied that are sensitive to it. For example, in 3T3 cells insulin increases cyclic GMP 5- to 20-fold at the concentrations which are teratogenic to the chicken (*73*).

I believe that the developmental path chosen by the mesodermal cells of the leg is determined by their concentrations of cyclic nucleotides. These also regulate further differentiation of chondrocyte and myoblast. Intracellular ion concentrations are also probably important, but the effects of the teratogenic compounds on cellular ionic concentrations are largely unknown. A high concentration of cyclic GMP (or a high ratio of cyclic GMP to cyclic AMP) is posited to trigger the differentiation of a presumptive muscle cell while high concentration of cyclic AMP (or low ratio of cyclic GMP to cyclic AMP) produces a presumptive bone cell. Thus, the stimulation of chondrocyte differentiation by 3-acetylpyridine and its inhibitory effects on myoblast differentiation may result from its ability to increase cyclic AMP in cells. Nicotinic acid may reverse the effects of 3-acetylpyridine by inhibiting the synthesis of cyclic AMP. Conversely, the ability of insulin to inhibit the normal development of bone and to reverse the effects of 3-acetylpyridine is postulated to result from its ability to increase cyclic GMP concentrations in cells.

Several observations support the above suggestion. Thyroxine, a hormone that increases cyclic AMP in sensitive cells (*97*) stimulates the differentiation of chondrocytes (*98*), and both thyroxine and dibutyryl cyclic AMP reverse the inhibition of chondrogenesis by hyaluronate in cell culture (*99*). Collagen, which can increase cyclic GMP concentrations (*73*), is required for myoblast differentiation in vitro (*22*). Similarly, the well-known trophic effects of acetylcholine and of insulin on muscle are in accord with this model. So is the decrease of adenylate cyclase activity preceding the terminal differentiation of cultured myoblasts. In a line of myoblasts conditionally unable to differentiate, the activity of adenylate cyclase does not change at the nonpermissive temperature (*100*). Finally, exogenous cyclic AMP appears to inhibit the differentiation of chicken myoblasts in vitro (*101*). It should be remembered that in melanoma cells (*41*), fibroblasts (*39*), or neurons (*39*) cyclic AMP stimulates the expression of differentiated functions. Thus, this effect of cyclic AMP on myoblasts, while in accord with the model, is not a result that would be expected on the basis of previous work with cyclic AMP.

Insulin, nicotinic acid, and 3-acetylpyridine appear to be convenient probes of the changes in cyclic nucleotide concentrations which are occurring in the affected tissues at the critical times when the teratogens are effective. Thus the sensitivity to insulin of a number of processes suggests that they depend upon a high ratio of cyclic AMP to cyclic GMP. In the case of the differentiation of muscle and bone the changes in the concentrations of internal messengers may be regulated by cell contact (*84*) since it is important for the normal differentiation of muscle and bone (*83*) and since contact with heterologous cells inhibits this differentiation (*102*).

Neurotransmitters and the Early Development of the Sea Urchin

Neurotransmitters occur in the early embryo. The involvement of neurotransmitters in the early development of the sea urchin has been investigated in several laboratories. The unfertilized egg is biochemically quiescent and DNA synthesis and cell division do not occur. Fertilization triggers many biochemical processes, including DNA synthesis, and cell division begins. Four synchronous cell divisions followed by six asynchronous divisions produce 1000 cells arranged in a hollow sphere, the blastula. After formation of the blastula, the rate of cell division slows considerably and the blastula hatches and becomes a free-swimming larva. A few hours after hatching, the primary mesenchyme cells (which will eventually form the skeleton of the larva) leave the ventral side of the blastula and enter a cavity within it, the blastocoel. They are soon followed by a major invagination of cells from the ventral side. These cells form a tube, the archenteron, which moves through the blastocoel, and after contacting the dorsal side of the larva, forms the digestive tract [see (*1*, *103*, *104*)].

Cell division and morphogenetic movements of cells are the main developmental events that occur in the early embryo. Buznikov and his collaborators (*32*, *105–107*) and Gustafson and Toneby (*33*, *104*) have accumulated considerable evidence that neurotransmitters are directly involved in both of these processes, even before neurons appear in the embryo. Neither group has suggested a biochemical basis for their action.

Several lines of evidence support the premise that neurotransmitters have a role in morphogenetic movements and cell division. Epinephrine, norepinephrine, and dopamine (*105*), serotonin (*32*, *105*, *107*, *108*), and acetylcholine (*105*, *109*) have been detected in the early sea urchin embryo by pharmacological, cytochemical, and chemical methods. Their concentrations change in close temporal association with both gastrulation and cell division (*32*, *105*, *107*). [Similar changes occur in the early embryos of a number of other phyla (*32*, *105*, *110*).] In the sea urchin early embryo, high concentrations of serotonin are localized in cells participating in morphogenetic movements (*107*).

Moderate concentrations of epinephrine, norepinephrine, isoproterenol, dopamine, or serotonin and various of their agonists inhibit the cell division and morphogenesis of embryos incubated in them (*33*, *106*, *111*); and added serotonin prevents the hatching of the blastula by preventing appearance of "hatching enzyme," even when it is applied as a single short treatment before the time of synthesis of the enzyme or its messenger RNA (*111*). Later in development serotonin causes abnormal skeletal development probably because of its effects on the movement of the primary mesenchyme cells (*33*).

Possible neurotransmitter regulation of the concentration of cyclic nucleotides. Antagonists of processes mediated by serotonin, acetylcholine, epinephrine, or dopamine also interfere (often at very low concentrations) with cell division and gastrulation. Some of these agents (and their pharmacological effects) are listed in Table 3. Many exert their physiological effects solely by interfering with cyclic nucleotide metabolism. These data indicate that catecholamines, such as epinephrine, affect development by a β-adrenergic mechanism involving the stimulation of cyclic AMP synthesis, since β-adrenergic agonists and antagonists interfere with development while α-adrenergic antagonists do not. The effects of tranquilizers such as chlorpro-

Table 3. The effects of pharmacological agents on development and on the metabolism of cyclic nucleotides.

Compound	Effect on development*	References for effect on	
		Development	Cyclic nucleotide metabolism
β-Adrenergic agonists or antagonists of the activation of adenylate cyclase			
Dichloroisoproterenol	++	(*33, 104*)	(*127, 129, 131, 146, 147*)
Dihydroergotamine	+	(*33*)	(*148*)
Propanolol	++	(*33*)	(*35, 128, 129, 149–151*)
Kö 592	++	(*33*)	(*150*)
Nylidrine	++	(*33*)	(*95, 147*)
Isoproterenol	+	(*104*)	(*35, 95, 149, 151*)
Dopamine	+	(*33*)	(*147, 152*)
Ephedrine	+	(*33*)	(*35*)
α-Adrenergic antagonists with little or no effect on the activation of adenylate cyclase			
Phentolamine	−	(*33*)	(*35, 128, 146, 149*)
Tolazololine	±	(*33*)	(*127, 131*)
Phenylephrine	±	(*33*)	(*151*)
Tranquilizers that inhibit the activation of adenylate cyclase by dopamine, epinephrine, and others			
Chloropromazine	++	(*33, 104*)	(*35, 152, 153*)
Fluphenazine	++	(*33, 104*)	(*152*)
Serotonin analog which activates adenylate cyclase			
Lysergic acid diethylamide	++	(*33*)	(*112*)
Muscarinic antagonist of acetylcholine that prevents increases in cyclic GMP			
Atropine	+	(*33*)	(*125, 134, 136*)
Nicotinic antagonists of acetylcholine that do not prevent increases in cyclic GMP			
Hexamethonium	−	(*33*)	(*136*)
Tetramethylammonium	−	(*33*)	(*136*)
Tubocurarine	−	(*33, 104*)	(*136*)

* Effect on development was scored as follows: ++, strong inhibition; +, inhibition; ± or −, little or no effect.

mazine support this interpretation. Similarly, the effects of exogenous serotonin and of LSD (lysergic acid diethylamide) on development could occur because of their stimulation of cyclic AMP synthesis (*112*). Acetylcholine may regulate developmental processes in the sea urchin embryo by increasing cyclic GMP since atropine, which inhibits acetylcholine-stimulated increases in cyclic GMP, interferes with development; but those anticholinergic agents that do not inhibit the increase in cyclic GMP do not inhibit development. The appropriate neurotransmitter often reverses the effects of its antagonist, an indication that the effects of the inhibitors are specific. For example, the effects of dichloroisoproterenol and alderlin on development are reversed by epinephrine (*105*).

The data in Table 3 suggest that neurotransmitters may be regulating cyclic nucleotide synthesis during the development of the sea urchin, but I can only make some general suggestions about the changes in ionic conditions which may also occur since available pharmacological data on invertebrates show that the ionic changes produced by a single transmitter can vary from cell to cell and even at different loci on the surface of the same cell (*113*).

The sea urchin embryo contains an adenylate cyclase, whose activity and characteristics change during development, and a guanylate cyclase which has not been similarly studied (*114*). Although the pharmacology of sea urchin adenylate cyclases has not been investigated, the cyclic AMP synthesis of other invertebrates is sensitive to neurotransmitters. For example, synthesis of cyclic AMP by adenylate cyclase is stimulated by dopamine and serotonin in the ganglion of a mollusk (*115*). Serotonin and its analog LSD stimulate the synthesis of cyclic AMP in an annelid and an insect (*112*). Epinephrine and serotonin stimulate the adenylate cyclase of protozoa and β-adrenergic antagonists can inhibit this stimulation (*37*). Studies on the effects of acetylcholine on cyclic nucleotide metabolism have been confined to mammalian tissues; but in every case in which it stimulates an atropine-sensitive receptor, the concentration of cyclic GMP is greatly increased while that of cyclic AMP may not change or may be slightly decreased. The changes in concentration of cyclic nucleotides which accompany the stimulation of β-adrenergic and atropine-sensitive receptors appear to be both necessary and sufficient for the physiological effects as experiments which cannot be adequately reviewed here have demonstrated. On the basis of the above analysis, the neurotransmitters appear to be important in development because they regulate the synthesis of cyclic nucleotides which, in turn, can guide morphogenetic movements by controlling the motility of cells or the adhesiveness of moving cells on the cellular substratum. I believe that the receptors for at least some of these neurotransmitters are located on intracellular membranes such as the nuclear membrane.

Mitosis, neurotransmitters, and cyclic nucleotides. The model allows specific predictions regarding a mechanism by which neurotransmitters and cyclic nucleotides could regulate mitosis in the embryo. On the assumptions (i) that the neurohumor-stimulated synthesis of cyclic nucleotides is a significant fraction of all synthesis and (ii) that the cellular concentrations of the cyclic nucleotides are proportional to the rates of synthesis, an interesting pattern emerges when data on changes in the concentration of neurotransmitters during mitosis are examined. These data [Fig. 1a, redrawn from (*107*)] show that an amazing sequence of changes of concentration of neurotransmitters could occur in the early embryo in synchrony with the phases of mitosis. Figure 1b presents estimates of the cyclic nucleotide concentrations in the dividing cells. This projection suggests that a number of pulsatile changes in the concentrations of cyclic nucleotides will occur during each mitosis. Since it is not possible to estimate the rates of cyclic nucleotide breakdown, I have drawn the duration of these changes to be identical to those of the "stimulating" neurotransmitter. The changes in the projected ratio of cyclic GMP to cyclic AMP are even more striking than those of the individual nucleotides. The highest ratio should occur during interphase and immediately be followed by the lowest ratio. As mitosis continues the amount of cyclic AMP drops, and that of cyclic GMP increases, so the ratio increases again during anaphase and reaches a maximum at the end of telophase and the beginning of interphase.

These postulated changes could be responsible for producing the striking changes in the physiology and morphology of the cell which are so characteristic of mitosis. They support a number of biochemical observations in mitosis. In this model the highest concentration of cyclic GMP during mito-

sis is believed to occur at the end of telophase and the beginning of interphase as a result of the large increase in acetylcholine concentration. The period of DNA synthesis in the early cleavage embryo of the sea urchin is coincident with this postulated maximum cyclic GMP concentration (*116*). Both acetylcholine and cyclic GMP have been shown to trigger DNA synthesis in other cells. Observations of the activity of adenylate and guanylate cyclases of the early embryo also agree with a correlation between a high ratio of cyclic GMP to cyclic AMP (or high cyclic GMP) and the period of DNA synthesis. Adenylate cyclase activity reaches a minimum during the first period of DNA synthesis after fertilization in the sea urchin (*114*). The egg (in which no DNA synthesis occurs) contains no guanylate cyclase; but the sperm is rich in this enzyme and cyclic GMP first appears in the egg after fertilization (*114*).

The concentration of cyclic GMP is suggested to drop to a low level during prophase and prometaphase when cyclic AMP is assumed to reach its highest concentration (Fig. 1b). This is the time of chromosome condensation and of the organization of the mitotic spindle (*116*). Phosphorylation of histone f1 has been suggested to play a role in chromosome condensation (*117*). There are a number of correlations between chromosome condensation and histone phosphorylation in cells ranging from the slime mold *Physarum polycephalum* to mammals (*117, 118*). Phosphorylation of histone f1 is catalyzed by cyclic AMP–activated protein kinases (*62–65*), whose activity in vivo is stimulated by cyclic AMP and by hormones that raise the cellular concentration of cyclic AMP (*46*). In many cells (*116, 117*) another burst of phosphorylation of histone f1 occurs during S phase, and in this regard it is interesting to note that the scheme presented in Fig. 1b suggests that an epinephrine-stimulated increase in cyclic AMP concentration occurs near the end of S phase. The relatively high level of cyclic AMP (and low level of cyclic GMP) which is suggested to exist in prometaphase and metaphase could be involved in the formation of the microtubular spindle. Protein kinases activated by cyclic AMP phosphorylate microtubule-subunit protein (*119*), and treatment of other cells with cyclic AMP, causes the assembly and stabilization of microtubules (*39*).

A reversal in the ratio of cyclic GMP to cyclic AMP probably occurs because of an increase in acetylcholine during anaphase and telophase. This is correlated with the reversal of the previous events of mitosis (that is, the spindle is disassembled and the condensation of the chromosomes is reversed). The question then arises as to whether cyclic GMP could be antagonizing the cellular events that may have been previously induced by cyclic AMP. Many antagonistic effects of cyclic GMP on processes activated by cyclic AMP are known (*35, 44, 58, 73*).

It is hard to deduce the nature of the changes in concentrations of ions which are occurring during the cell cycle. However, I suspect that the amount of available K^+ and Ca^{2+} increases during periods of high acetylcholine content (anaphase and telophase-interphase). The distribution of Na^+ may also change, perhaps decreasing in amount, at this time. These changes could help produce the biochemical and morphological events which are characteristic of these periods including the depolymerization of microtubules (*120*) and the regulation of DNA synthesis.

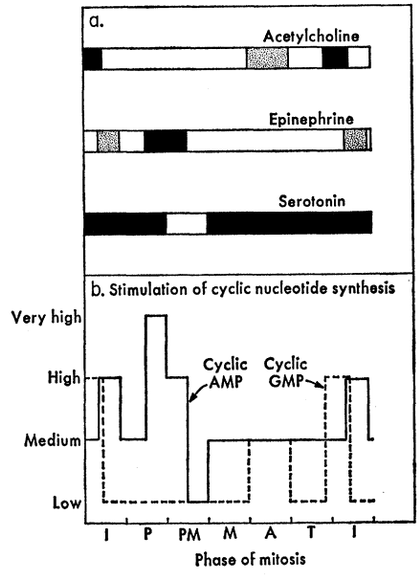

Fig. 1. (a) Neurotransmitter concentrations in the cells of early embryos as a function of their stage in mitosis. This figure, redrawn from (*107*), presents the measurements of neurotransmitters made by Buznikov *et al.* Transmitter concentrations in micrograms per 10^6 eggs are: solid line, serotonin (3 to 5), epinephrine (1.0 to 1.5), acetylcholine (5 to 7); dotted line, epinephrine (0.5 to 0.7), acetylcholine (2 to 4); blank line, serotonin (≤ 1), epinephrine (0.4), acetylcholine (≤ 2). (b) Postulated effects of the endogenous neurotransmitters on cyclic nucleotide synthesis as a function of stage in mitosis. Serotonin and epinephrine are assumed to stimulate the synthesis of cyclic AMP while acetylcholine is assumed to stimulate cyclic GMP synthesis. The abbreviations for the phases of mitosis on the abscissa are *I*, interphase; *P*, prophase; *PM*, prometaphase; *M*, metaphase; *A*, anaphase; and *T*, telophase.

This interpretation suggests that treatment of cells with high concentrations of cyclic AMP or cyclic GMP prevents cell division by preventing the normal triggering of cellular events that depend on the ratio of cyclic GMP to cyclic AMP or on their absolute concentrations in the cell, and therefore that cyclic AMP and cyclic GMP should block mitosis at different stages. Changes in the rates of cyclic nucleotide accumulation should be detectable during mitosis in the early cleavage embryo. Furthermore, it may be possible to move cells through mitosis a step at a time by regulating cyclic AMP and cyclic GMP under appropriate ionic conditions.

The fact that biochemical observations of mitosis in a variety of cells are in accord with the scheme suggested for the sea urchin embryo suggests that similar series of changes of the concentration of cyclic AMP and of cyclic GMP could regulate traversal of the mitotic cycle in other cells. One such cell may be *Tetrahymena pyriformis* where changes in the cellular concentration of acetylcholine occur during the cell cycle and in which adrenergic inhibitors inhibit mitosis (*121*). Fluctuations in cyclic AMP content occur during the cell cycle (*122*), but no investigation of cyclic AMP or cyclic GMP concentrations during mitosis itself has been published.

The suggestion that cyclic nucleotides regulate traversal of the cell cycle may appear to conflict with the theme of my article, that cyclic nucleotides are used to regulate other developmental phenomena including cell differentiation and morphogenesis. This mechanism requires that the regulation of other developmental phenomena be uncoupled from regulation of mitosis. The well-known uncoupling of the expression of "differentiated" characteristics from cell division in many types of cells agrees with this requirement. Cyclic AMP induces some enzymes only during specific parts of the cell cycle (*123*). Finally, the production of differentiated functions in cultured cells by cyclic AMP (*39, 41*) is often accompanied by inhibition of cell division.

Conclusion

The hypothesis that physiological and developmental regulatory mechanisms are similar has been presented. Well-known developmental systems chosen illustrate the capability of the model to suggest a simple mechanism underlying the effects on development of a diverse group of chemicals. This hypothesis might be applied to other systems including the induction of the lens, limb regeneration, and the induction of the head of hydra (*124*).

I have proposed this hypothesis not only because it permits consideration of a complex and varied array of experimental observations as reflections of a simple basic biochemical mechanism, but because recent technical advances in instrumentation and methods allow it to be directly tested. The fluorescent antibody method for the cytochemical measurement of cyclic nucleotides provides a means for investigating changes in the concentrations of cyclic nucleotides in developing cells and could also be used to detect neurotransmitters in developing cells. Similarly, the scanning electron microscope in the emitted x-ray mode provides a method for measuring changes in the content and distribution of cations within developing cells.

The hypothesis presented here suggests pleasing asceticism on the part of eukaryotes. It suggests that simple derivatives of metabolites, including neurotransmitters and cyclic nucleotides, are linked together as regulatory molecules throughout the eukaryotes. The neurotransmitters are suggested to have a more general role in information transmission in eukaryotes than is generally accepted. They are hypothesized to have progressed during evolution from being intracellular messengers to a role as intercellular messengers for the relatively slow communication of developmental informaton; and, finally, this process has culminated with their participation in the rapid intercellular communication mediated by nerves. The thought that the complex pictures of physiological regulation and of the construction of a complex multicellular organism like man might be painted with so few colors is quite satisfying.

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