CONTROL OF CHONDROGENESIS IN THE EMBRYO

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ABSTRACT Control of chondrogenesis in the embryo involves mechanisms which induce certain cells to synthesize chondroitin sulfate, as well as mechanisms which regulate the continued production of these molecules in differentiated chondrocytes. The embryonic spinal cord and notochord induce somite cells to chondrify. Interaction between these inducers and somite cells, however, is not sufficient in itself to transform the latter into chondrocytes; there must be additional reactions. The notion that induction simply involves the transmission of information-rich molecules like RNA or protein from inducers to somite cells is thought unlikely. When differentiated 10-day chondrocytes with all the enzymes and genetic information for the synthesis of chondroitin sulfate are removed from their mucopolysaccharide matrix and cultured as isolated cells *in vitro*, they rapidly transform from spherical cells to stellate ones. Correlated with this change in shape and increase in surface area of cell membrane is the cessation of the production of chondroitin sulfate. The stellate cells are induced to synthesize DNA and multiply; their progeny, though permitted to resume their originally spherical shape, do not differentiate into recognizable chondrocytes again. Experiments of this kind suggest the presence of metabolic controls located in the cytoplasm and/or cell membrane.

INTRODUCTION

Microscopists have studied cartilage for many years. Chondrocytes are readily recognized by their somewhat spherical shape and their surrounding hyaline matrix which stains metachromatically. In the past decade this matrix has been subjected to a more rigorous analysis and its major polysaccharide components identified as chondroitin sulfates A and C (Meyer, 1950; Davidson and Meyer, 1955). Only more recently has the protein associated with the chondroitin sulfate received attention (Malawista and Schubert, 1958; Muir, 1958; Matthews and Lozaityte, 1958).

A comprehensive review of chondrogenesis during development, then, should consider the structure of the cartilage cell as well as its biochemical activities. For if cells are not just "bags of enzymes" an analysis of chondrogenesis should show how the biosynthetic activities depend upon the structure of the cell and, conversely, how its biochemical activities determine its structure.

As the embryo develops, some cells are induced to synthesize chondroitin sulfate

and collagen whereas others are induced to synthesize myosin, or hemoglobin, or cortisone, or other specialized molecules. Over-all control of chondrogenesis must involve mechanisms which divert certain cells into initiating the production of chondroitin sulfate and mechanisms which sustain chondrogenic activity in the functional chondrocyte. Put in other terms, the regulation of chondrogenesis is concerned with (*a*) the environmental cues activating the genetic information stored within prospective chondroblasts, and (*b*) the genetic controls which keep a chondrocyte dedicated to the production of chondroitin sulfate.

Since the experimental data have been recently reviewed in detail (Holtzer, 1961, 1963), the following will be largely speculative, stressing the controversial aspects of the material as well as emphasizing the pitfalls of working with embryonic induction systems. This concern with pitfalls may be timely when many are applying, perhaps not too critically, some of the techniques of biochemistry and the concepts of microbial genetics to problems of cell differentiation.

BACKGROUND

In all vertebrates dividing somite cells eventually cease to synthesize DNA and differentiate into the vertebral cartilages and muscles of the trunk. Extirpation of the embryonic spinal cord and notochord at the proper stage does not prevent the progeny of somite cells from differentiating into muscle, but it does preclude their differentiating into cartilage. If a piece of spinal cord or notochord is grafted, *in vivo,* among somite cells which normally would have formed muscle, such cells are diverted into forming chondrocytes. Cells which would have synthesized myosin and actin are shunted into synthesizing chondroitin sulfate and collagen by interacting with spinal cord or notochord. On the basis of these experiments it is clear that the spinal cord and notochord induce the *progeny* of somite cells to differentiate into chondrocytes (Holtzer and Detwiler, 1953; Avery, Chow, and Holtzer, 1956).

Induction having been observed on a tissue level, we now would like to learn more of what is involved on a cellular and molecular level. And we might anticipate our findings by saying that what appears as a single event morphologically is a multiplicity of events on a cellular and molecular level.

IN VITRO ANALYSIS OF CARTILAGE INDUCTION

When randomized stage 18 somites from a chick embryo (Hamburger and Hamilton, 1951) are grown on a clot they fuse and the cells multiply, forming a sheet of mesenchyme criss-crossed with immature myotubes. Cartilage will not differentiate in these cultures though they may be kept for over 2 weeks. Similar clusters of somites plus a piece of spinal cord or notochord form cartilage in addition to mesenchyme and muscle cells, generally 4 days after the cultures are set up. From this it is concluded that when spinal cord or notochord is added to the cultures, they affect the eventual synthetic behavior of somite cells as they do *in vivo.*

The induction of chondrogenesis in a population of somite cells is a relatively specific tissue interaction (Holtzer, 1951; Avery, Chow, and Holtzer, 1955; Grobstein and Holtzer, 1956; Lash, Holtzer, and Holtzer, 1957). Many tissues and agents which induce salamander ectoderm (Holtfreter and Hamburger, 1955) or which induce chondrogenesis in mature animals proved incapable of stimulating chondrogenesis in somite cells. As stressed previously, however, the specificity of the spinal cord or notochord is relative and in the embryo there must be many cartilage inducers. The spinal cord and notochord do not induce the cartilaginous appendages, girdles, ribs, trachea, or hemal arches, not to mention ectopic nests of cartilage in the bladder, liver, blood vessels, or muscles of mature animals.

Cartilage is not detected cytologically, primarily by its characteristic metachromasia, until the 4th day of culture. Question: Must the spinal cord interact with somite cells for the entire 4 days or will briefer periods of interaction suffice? To test this, spinal cord was explanted on one side of a millipore filter, somites on the other. After 2, 5, 15, etc., hours of transfilter interaction, the spinal cord was removed and the somites on the other side of the filter inspected for cartilage 4 days later. The results of these experiments are shown in Table I. At least 10 hours of

TABLE I

TRANSFILTER INDUCTION

Time somites exposed to spinal cord	No. of cultures	
	Cartilage	No cartilage
hrs.		
2–3	0	15
5	0	25
10	12	28
16	18	20
24–48	33	6

Consult text for details of experiment (from Lash, Holtzer, and Holtzer, 1957).

transfilter interaction are necessary if cartilage is to be induced in half the cultures 4 days later. Prolonging the transfilter interaction resulted in a higher proportion of inductions. Ten hours is a maximum figure, for several hours are required for the tissues to adhere to the filter and probably several more for material to be carried, or to diffuse, through the cellulose acetate membrane. The time during which a postulated "cartilage-inducing molecule" from the spinal cord actually acts on responsive somite cells requires at most a few hours and may in fact take but minutes. In both *in vivo* and *in vitro* situations the inducers act on competent somite cells and approximately 4 days later recognizable chondrocytes appear. This period has been likened to the eclipse period following phage infection (Holtzer, 1961,

1963). It is obvious that an understanding of induction requires a better understanding of what is happening to prospective chondroblasts during this 4 day silent period (Avery, Chow, and Holtzer, 1956; Lash, Holtzer, and Whitehouse, 1960).

AT WHAT STAGE ARE THERE INDUCED CELLS IN SOMITES?

The thousands of cells in a stage 18 somite are a very heterogeneous group with respect to their chondrogenic and myogenic capabilities (Holtzer, Marshall, and Finck, 1957). Prior to culturing it is likely that an indeterminate number of stage 18 somite cells have already interacted with the *in vivo* inducers. Whether or not cells induced *in vivo* express their chondrogenic capacities 4 days later depends upon where and how they are grown after being removed from the embryo. As shown in Table II the same number of somites of a given stage are more likely to form cartilage if grown on the chorioallantoic membrane than if grown on nutrient agar. In turn equivalent somites will more frequently behave as induced if cultured on agar than if they are grown on a fibrin clot. Somites growing on top of a clot spread, approximating a monolayer culture, whereas when grown on agar they remain compacted. Apparently spreading is inimical to chondrogenesis. (See page 247 of this paper.)

Still another phenomenon is suggested in the data of Table II. Ten stage 16 somites grown on agar do not form cartilage; 30 stage 16 somites grown on agar form cartilage in 90 per cent of the cultures. After 7 days of growth there are relatively few surviving cells in the cultures established with 10 stage 16 somites. On the other hand cultures of 30 stage 16 somites increase in cell number and differentiate into cartilage. These simple experiments which permit the cells to behave as "induced" in one growth situation, but as "uninduced" in another suggest that the action of inducers on the somite cells is not by itself sufficient to transform the latter into chondrocytes. Whatever the nature of the interaction between spinal cord and somites, additional events, such as compacting of the somite cells and their multiplication, must intervene before the somite cell is a recognizable chondrocyte.

The ease with which the induced state may be "canceled" is indicated by the following: Clusters of 10 stage 19 somites form sizable nodules of cartilage in 100 per cent of the cultures 4 days after the cultures are set up. If identical somite cells are made into a cell suspension by means of trypsin (Moscona, 1953) and then cultured, cartilage does not form in any of the cultures. Failure of suspended somite cells to differentiate into cartilage is not due to lack of viability, for these cells divide more rapidly than do the cells in somite clusters.

In another series clusters of stage 14 somites were cultured as a mesenchymal sheet on a plasma clot for 3 days. The cells were harvested, compacted, and recultured with fresh spinal cord or notochord. Cartilage did not develop in these cultures. Apparently somite cells must be in a certain competent state to respond to the inducers. Culturing them as cells in monolayers for 3 days results in their losing

TABLE II

CHONDROGENIC ACTIVITY IN SOMITES OF DIFFERENT STAGES, GROWN UNDER DIFFERENT CONDITIONS, AND CULTURED WITH AND WITHOUT INDUCERS

Stage of somites	Where grown	Per cent with cartilage: Somites alone	Per cent with cartilage: Somites plus inducers
20 randomized	CAM graft	100	100
20 randomized	ON clot	100	96
19 randomized	On agar	100	—
19 dissociated and aggregated	On agar	0	95
18 randomized	CAM graft	90	90
18 randomized	On agar	75	—
18 contiguous strips	On clot	45	95
18 randomized	On lens paper	0	85
18 dissociated and aggregated	On agar	0	60
16 contiguous strips	CAM	80	95
16 randomized	CAM	33	90
16 randomized	On clot	0	85
16 randomized	On agar	0	—
*16 randomized—30 somites	On agar	90	—
14 contiguous strips	CAM graft	42	90
14 randomized	CAM graft	0	90
14 randomized	On clot	0	80
14 randomized	On agar	0	—
*14 randomized—30 somites	On agar	20	—
12 randomized	CAM graft	0	80
12 contiguous strips	On agar	0	85
12 randomized	On agar	0	90
12 randomized	On clot	0	—
*12 randomized—30 somites	On agar	0	—

When possible the anterior somites were discarded and only the posterior somites tested. Fibrin clots were used with or without feeding medium. The agar was prepared by mixing nutrient medium with 2 per cent agar. CAM stands for chorioallantoic membrane, and randomized refers to the fact that the individual somites were clustered without regard to their anterior-posterior position in the trunk. Except where noted by asterisks 10 somites were used in each culture or graft.

their state of inducibility (Nameroff, Holtzer, and Holtzer, unpublished data).

Taken together these several experiments are not compatible with the simple notion that the spinal cord introduces an "information-rich" molecule into the uninstructed somite cell after which the latter is "genetically" destined to transform into a chondrocyte. Only previously instructed somite cells can even respond to the spinal cord or notochord. And after this inductive act, the responding somite cells must be subjected to additional "inductive acts" if they are eventually to transform into chondrocytes.

ISOLATION OF THE INDUCING FACTOR

It has been reported (Lash, Hommes, and Zilliken, 1962) that a fraction from a cold perchloric acid extract of spinal cord and notochord induces chondrogenesis. This fraction (Hommes, van Leewen, and Zilliken, 1962; Zilliken, 1963) consisting of polypeptides, nucleotides, and sugars is said to contain the inducer molecule. Strudel (1962) reports that a saline extract of spinal cord or notochord induces somites to chondrify. In view of the varying responses of the somites depending upon where and how they are grown (see Table II) it is difficult to evaluate these two reports, which use these *in vitro* systems to test their extracts. For example, as stated earlier, 10 stage 16 somite cells by themselves dwindle in number and do not form cartilage, whereas 30 stage 16 somite cells increase in number and form cartilage. If extracts added to cultures of 10 stage 16 somites chondrify, is this because the extract simulates the inducing action of the spinal cord or because it promotes the survival of cells already induced? Operationally, with our current techniques, this is a difficult question to grapple with. As an assay system, the *in vitro* preparation is unequivocal *on a tissue level,* in stating whether or not somite cells transform into chondrocytes; but it cannot be used to distinguish unambiguously between the action of "the inducing molecule" and the action of non-specific growth-promoting substances so important in all tissue cultures.

CHEMODIFFERENTIATION AND CYTODIFFERENTIATION

In earlier work (Holtzer, 1961, 1963, and 1964) it was stressed that the chondroitin sulfates were absent in early somite cells and that the transformation into histologically recognizable chondroblasts is correlated with the synthesis of these particular acid mucopolysaccharides. This view will be untenable if the recent findings of Franco-Browder, DeRydt, and Dorfman (1963) are confirmed. These investigators report the presence of "chondroitin sulfate" in stage 12 embroys, the earliest they analyzed. There are, however, several observations in this work that are difficult to evaluate. There are many different kinds of acid mucopolysaccharides in embryonic tissues associated with cell membranes, mitotic spindle, nucleus, and cytoplasmic inclusions (Immers, 1956, 1958; Tyler, 1961). That the isolation techniques used by

Franco-Browder and coworkers could distinguish between these polysaccharides is not likely. The material isolated from the young chick embryos binds acridine orange, is hydrolyzed by RNAase and hyaluronidase, but whether it has anything to do with chondrogenesis is not at all clear. That it is found in extraembryonic membranes and skin (Franco-Browder, personal communication) as well as in the spinal cord and somites, makes its connection with chondrogenesis difficult to comprehend. Also difficult to reconcile with the known biology of the system is that there is as much of the undersulfated "chondroitin sulfate" in the anterior somites as in the posterior somites. The basement membrane of the skin, the sheaths of the notochord, and the meninges of the spinal cord all stain metachromatically with toluidine blue, and these same regions stain positively for acid mucopolysaccharides when treated with periodic acid–Schiff reagent. Acridine orange is strongly bound by the DNA and RNA of all cells and is strongly bound by the same areas reacting metachromatically with toluidine blue or positively with PAS; in addition all cell membranes bind the fluorescent dye to a slight degree. The significance of the findings of Franco-Browder *et al.* will become more apparent only when they demonstrate that the negatively charged molecule they have extracted is related to the chondroitin sulfate of chondrocytes and that it is located in the somite cells which will transform into chondrocytes.

SOME PROPERTIES OF THE FUNCTIONAL CHONDROCYTE

To learn more of the chondrocyte on a genetic and biosynthetic level we turned to 10-day embryonic cartilages. These cartilages, indistinguishable under the microscope from mature cartilage, consist of chondrocytes actively synthesizing chondroitin sulfate. The individual cells are surrounded by considerable metachromatic matrix. By digesting the cartilages with trypsin a cell suspension of freshly liberated chondrocytes is obtained virtually free of contaminating cells. With these cells it is possible to do some simple "genetic" experiments. One may ask such questions as, is the differentiated state of a chondrocyte transmitted *via* mitotic divisions to successive generations and is the capacity to synthesize chondroitin sulfate an inheritable trait?

Freshly liberated chondrocytes were spun down into pellets and organ-cultured on agar in the presence of $^{35}SO_4$. Six days later representative pellets were fixed, sectioned, radioautographed, and stained. Companion pellets were extracted for chondroitin sulfate and the amount of labeled isotope determined. The results of these experiments are shown in the first line of Table III. The mitotic activity was determined by rearing pellets for 24 hour periods in labeled thymidine (Table IV). From these results it can be concluded that: (*a*) The synthetic activities of freshly liberated chondrocytes are not impaired by the trypsin liberation procedures; (*b*) the initial population was not contaminated by non-chondrogenic cells; and (*c*) when

TABLE III

AMOUNT OF S^{35} BOUND BY CELLS IN PELLETS AFTER VARYING PERIODS AS CELLS IN MONOLAYERS

Time in monolayers	Total counts/min./ 5×10^6 cells grown in pellets	Chondrocytes in organ culture
days		*per cent*
0	505,233	98
5	265,851	40–60
11	2,144	0
21	1,806	0
26	2,205	0

All cells were derived from the same strain, were exposed to the same amount of $S^{35}O_4$, and were extracted in the same way. Estimate of per cent of chondrocytes in the pellets is approximate. Each measurement is based on the mean value of 4 or 5 cultures. Pellets were cultured for 6 days (from Holtzer, Abbott, Lash, and Holtzer, 1960).

TABLE IV

CHONDROCYTE LABELING EXPERIMENTS

Time	Intact vertebrae	Pellets	Monolayers
hrs.	*per cent*	*per cent*	*per cent*
0–24	18	16	18
24–48	22	12	77
48–72	19	10	78
72–96	4	15	62

The percentage of chondrocytes incorporating tritium-labeled thymidine during a 24 hour period in culture. These are average values based on over 40 experiments during a 4 month period (from Stockdale, Abbott, Holtzer, and Holtzer, 1963).

cultured in pellets the progeny of slowly dividing chondrocytes may differentiate into cartilage cells.

Whether the progeny of liberated chondrocytes *first reared in monolayers* and then organ-cultured form chondrocytes depends upon how long they are first grown in monolayers (Holtzer, Abbott, Lash, and Holtzer, 1960; Stockdale, Abbott, Holtzer, and Holtzer, 1963). Chondrocytes grown in monolayers synthesize DNA and divide more frequently than do similar cells *in vivo* or in pellets (Table IV). When the progeny of chondrocytes which have multiplied in monolayer cultures for 5 or 6 generations are organ-cultured as pellets, they do not synthesize the mucopolysaccharides characteristic of chondrocytes. Whether this loss is reversible or irreversible, whether it is a function of the number of mitotic divisions the cells have undergone, or whether under the conditions of monolayer growth they cannot retain essential metabolites (Eagle and Piez, 1962) remains to be determined.

Dedifferentiated chondrocytes cannot be induced to chondrify by growing them with spinal cord or notochord. From this it is concluded that dedifferentiated chondrocytes do not revert to their embryonic somitic condition.

Kuroda (1963) has found that liberated cartilage cells grown in monolayers exhibit a rapid decline in mutual cohesiveness. This loss correlates well with the decline in the synthesis of chondroitin sulfate.

These experiments suggest that the differentiated state of a tissue cell need not be transmitted by mitotic divisions to all its progeny.

INTERDEPENDENCE OF SHAPE AND FUNCTION OF CHONDROCYTES

After 5 or 6 generations in monolayer cultures the progeny of chondrocytes, spun down into pellets and organ-cultured, do not synthesize chondroitin sulfate. A question of interest is when and under what conditions the liberated chondrocytes in monolayers *first cease* producing sulfated mucopolysaccharides. To answer this monolayers of liberated chondrocytes were reared as isolated cells in the presence of $^{35}SO_4$. The cultures were sacrificed after 1, 5, 15, etc., hours, radioautographed, stained with toluidine blue, and the grains over the cells counted. The results are shown in Table V (from Pettengill and Holtzer, 1963). Immediately after liberation all the cells are spherical and there is no metachromatic material associated with the cell membranes. Within 5 hours in culture many of the rounded cells are coated with metachromatic matrix. Grains are invariably present in the emulsion above the rounded, metachromatic cells. By 15 hours there are fewer rounded cells but they have more metachromatic material on their cell surface and the number of grains over such cells has increased. The proportion of stellate to rounded cells has gone up and the stellate cells lack metachromatic material and have fewer grains. Empty metachromatic capsules free of cellular debris are found in 15 hour, and more frequently in 24 hour, cultures. In 15 and 24 hour cultures many cells are observed crawling out of the metachromatic capsules. Invariably the vacated metachromatic "ghosts" have grains above them, demonstrating their recent *in vitro* synthesis. After 36 hours all rounded cells with or without metachromatic material have disappeared though ghosts are present. By pulsing with $^{35}SO_4$ for 3 hours it can be shown that ghosts do not incorporate labeled sulfate (Abbott and Holtzer, unpublished data).

The apparent emergence of two cell types in the first 24 hours of culture is not due to cell selection among cells of different types, as may be determined by the absence of mitotic activity (Table V). The stellate cells are derived by transformation of the rounded chondrocytes. Associated with this transformation in shape is the cessation of sulfate uptake and the cessation of the deposition of metachromatic matrix. Isolated rounded cells cannot maintain themselves in these monolayer cultures and by 24 hours all have transformed into stellate cells, shedding their capsules, if they had formed them. Stellate cells, though not actively synthesizing

TABLE V

Time in culture	Rounded cells	Stellate cells	Metaphase plates	No. of grains/100 rounded cells	No. of grains/100 stellate cells	No. of grains/100 cells in clusters
hrs.	*per cent*	*per cent*	*per cent*			
1	100	0	0.01	180 (0–8)	—	—
5	95	5	0.01	1078 (0–42)	122 (0–7)	1551
15	22	78	0.01	4807 (16–82)	594 (0–21)	+++
24	7	93	0.05	4022 (13–76)	828 (0–28)	+++
48	2	98	6.0	Ghosts 26–72	535 (0–16)	+++

Freshly liberated cartilage cells were grown in the presence of $^{35}SO_4$ for varying periods, radioautographed, and the grains per 100 rounded and per 100 stellate cells counted. The figures in parentheses below indicate the distribution. Ghosts refer to the shed metachromatic matrix material. The +++ in the column of grains/100 cells in clusters denotes the fact that there were too many grains to count accurately (from Pettengill and Holtzer, 1963).

chondroitin sulfate, retain the capacity to make such molecules up to approximately the 8th day of culture (Stockdale, Abbott, Holtzer, and Holtzer, 1963).

The majority of 10-day chondrocytes *in vivo* are postmitotic cells. When liberated these cells transform into stellate cells, cease synthesizing chondroitin sulfate, and *start* synthesizing DNA. By the 48th hour of culture approximately 95 per cent of the stellate cells will have divided at least once. In pulse experiments cells have been exposed to labeled sulfate and tritiated thymidine. The vast majority of monodisperse cells which incorporate thymidine do not take up S^{35}-sulfate (Abbott and Holtzer, unpublished observations). Clearly it is important to learn more of the relationship between the chondrocyte's ability to synthesize DNA and its capacity to synthesize chondroitin sulfate (Holtzer, 1964).

Individual spherical chondrocytes may transform into stellate cells within as short a period as 4 hours. On the assumption that this change in shape is not accompanied by any significant change in volume, then the increase in cell membrane that must accompany this transformation can be estimated. If the volume of a rounded cell with a radius of 10 μ is held constant and the cell converted into a flattened cylinder 1 μ in thickness, then the surface of cell membrane increases over fivefold. This rapid recruitment of cell membrane, whether by the unfolding or utilization of preexisting cell membrane or by *de novo* synthesis, must impose considerable strains upon the metabolism of the cell.

SUMMARY AND CONCLUSIONS

There is no such thing as an uninstructed cell and competent somite cells must

possess specific information in order to respond to the inducing action of spinal cord or notochord. Many conditions may limit the realization of the interaction between spinal cord or notochord and somite cells. Between this interaction and the appearance of the first chondrocyte there are many chemical events, any one of which, if inhibited, would block chondrogenesis. The initiation of each of these sequential events could on a molecular level properly be called an inductive event. While a little is known of induction on a tissue level, on a cellular, a biochemical, or a genetic level, we must acknowledge that in spite of many attractive speculations and models, nothing is known.

Liberated chondrocytes grown in pellets retain their spherical shape and continue to synthesize chondroitin sulfate. Similar cells in monolayers in a few hours transform into stellate cells, cease to synthesize chondroitin sulfate, and begin to synthesize DNA. Cells with identical genetic information, and, at least initially, with the same enzymes, can be made to engage in different metabolic activities; *i.e.*, either to make chondroitin sulfate or all those other kinds of molecules required for cell multiplication. The rapidity with which a "trivial" change in the shape of a cell induces or reflects changes in biosynthetic activities should be remembered before attributing induction to an exogenous protein or RNA.

Some of the speculations in this paper will be easier to disprove than others. But given the flux in the field of cell differentiation I would like to conclude with a few predictions: (*a*) Neither embryonic nor mature cells grown in the presence of tissue-specific RNA or RNP will transform into the type of cell from which the nucleic acid was obtained. There is as yet no reliable evidence that messenger RNA from one type of tissue cell can, by entering another type of cell, alter the latter's characteristic metabolic activity. (*b*) The molecules maintaining a differentiated cartilage or muscle cell are probably not the same molecules which initially channeled the cell into becoming a chondroblast or myoblast. (*c*) A tissue cell synthesizing molecules like chondroitin sulfate or myosin must have the cytological features of cartilage or muscle cells. Cartilage or muscle cells may not at all times be actively synthesizing their respective unique molecules, but when they do they must exhibit characteristic structures. (*d*) Knowledge of cell differentiation on a molecular level awaits a better understanding of what is happening to cells when they are acquiring the pathways necessary to synthesize chondroitin sulfate. The real gap in understanding cell differentiation is not so much in what the differentiated cell is doing to make its unique molecules, but in what it and its precursor cell did to prepare it for its unique biosynthetic activity.

Dr. Holtzer is a Research Career Development Awardee (GM-K3-2970).

This investigation was supported in part by Research Grants B-493 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health and NSF G-14123 from the National Science Foundation.

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