THE LOSS OF PHENOTYPIC TRAITS BY DIFFERENTIATED CELLS

III. The Reversible Behavior of Chondrocytes in Primary Cultures

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ABSTRACT

Observations were made on the behavior of chondrocytes grown under various conditions in vitro. The chondrocytes in 10-day embryonic chick vertebrae were grown as cultures of intact vertebrae, as pellets of chondrocytes liberated from their matrix, and as monodispersed cells plated out on plasma clots. Cartilage matrix was stained metachromatically with toluidine blue. Radioautographs were made of incorporated H^3-thymidine, H^3-proline, and S^{35}-sulfate to determine the extent of DNA synthesis, collagen synthesis, and chondroitin sulfate synthesis, respectively. Chondrocytes in intact vertebrae or in pellets are rounded and actively synthesizing chondroitin sulfate and collagen. There is little DNA synthesis by cells in either vertebrae or pellets. Chondrocytes grown as monodisperse cells rapidly cease synthesizing cytologically detectable chondroitin sulfate and are induced to synthesize DNA and divide. There is a change in the shape of these chondrocytes from a rounded to a more stellate condition which accompanies the shift in metabolic activity. Conversely, when the cells attain a certain cell density, they reacquire a rounded shape, cease dividing, and again synthesize chondroitin sulfate. Clusters of chondrocytes synthesize more chondroitin sulfate than isolated chondrocytes. It is concluded that most chondrocytes synthesizing chondroitin sulfate do not concurrently synthesize DNA. Interaction between associated chondrocytes is important in inducing and maintaining chondroitin sulfate synthesis in genetically determined chondrocytes. Failure of interaction between chondrocytes leads to DNA synthesis and cell multiplication.

INTRODUCTION

Specialized cells which make unique molecules such as myosin, thyroxin, or hemoglobin normally neither synthesize DNA nor divide (Heidenhain, 1911; Peter, 1929; Cowdry, 1932; Holtzer, 1961, 1964). Such cells behave as if the synthetic pathways for DNA, and probably those for the mitotic spindle proteins and the chromosomal proteins as well, are turned off in favor of the pathways for producing unique somatic molecules. This shift from division to nondivision and specialization presents a basic problem in cell differentiation about which very little is known. Similarly, little is known of factors regulating the shift from specialization to division observed in regeneration, wound repair, tumorigenesis, and many in vitro situations (Ebling, 1924; Doljansky, 1930; Bloom, 1937; Weiss, 1950; Needham, 1952; Nowell, 1960; Holtzer et al., 1960; Ebner, Hageman, and Larson,

1961; Stockdale and Holtzer, 1962; Whittaker, 1963; Stevens, 1964; Moore, 1964).

The present study deals with some aspects of these seemingly alternate states of cell activity. Chondrocytes were used because they constitute a readily available pure population of cells which elaborate a matrix consisting largely of chondroitin sulfate and collagen, both of which can be recognized cytologically. The behavior of chondrocytes was analyzed under a variety of culture conditions and after exposure to H^3-thymidine, S^{35}-sulfate, and H^3-proline.

It was found that chondrocytes can rapidly shut off chondroitin sulfate synthesis and initiate instead the production of those various kinds of molecules required for cell division (Mazia, 1961). A change in cell shape from round to stellate is correlated with this metabolic switch. Conversely, as cell density increases, the stellate cells change back to rounded cells, cease making DNA, and may resume the synthesis of chondroitin sulfate.

MATERIALS AND METHODS

TISSUE CULTURE: The procedures used are modifications of those described by Holtzer et al. (1960). The ventral half of the vertebral column was removed from 10-day white Leghorn chick embryos. Cartilages were cleaned of all adhering tissues, cut into 1-mm^3 pieces, and incubated for 2½ hr at 37°C in 1% trypsin (Difco 250) dissolved in Ca-Mg-free Simms' solution (Moscona, 1952). Cells were liberated from their matrix by flushing the cartilages through a Pasteur pipette. The culture media used were: 1) 2 parts horse serum, 2 parts balanced salt solution (Simms'), and 1 part embryo extract (2:2:1); and 2) 8.8 parts Eagle's minimal essential medium, 1 part horse serum, and 0.1 part L-glutamine (MEM). Both media contained 0.1% penicillin-streptomycin solution. For dispersed cell cultures, cells were grown in or on thin plasma clots (1 plasma: 1 embryo extract on coverslips in Leighton tubes containing 1 ml of medium). Short-term organ cultures of liberated cells were made by centrifuging aliquots of cells and growing them as small pellets in the bottom of centrifuge tubes or in the bottom of embryological watch glasses. Intact vertebrae were also cultured in the bottom of centrifuge tubes or watch glasses (de la Haba and Holtzer, 1965).

HISTOLOGY: Cultures were fixed in acetic alcohol (1 part acetic acid: 3 parts absolute alcohol) and postfixed in either formol-saline (10% formalin in balanced salt) or glutaraldehyde (1% in 0.1 M phosphate buffer pH 7.8). Pellets and intact vertebrae were embedded and sectioned at 4 μ. Toluidine blue was used to stain cartilage matrix metachromatically.

AUTORADIOGRAPHY: Tissues were exposed to the sodium salt of S^{35}-sulfate (5 μc/1 ml, Abbott Laboratories, North Chicago, Illinois), H^3-thymidine (0.5 to 1.0 μc/1 ml, Schwartz Bio Research Inc., Mt. Vernon, New York; sp. act. 6.5 c/mmole), or H^3-proline (0.5 μc/1 ml, Schwartz Bio Research Inc., sp act 1.15 c/mmole) at various times during the culture period. Coverslip cultures and 4-μ sections of pellets and intact vertebrae were covered with NTB-3 Kodak emulsion (Messier et al., 1957). Slides were developed for 5 to 7 min at 25°C.

RESULTS

Characteristics of Chondrocytes in Intact Vertebrae

Cartilage cells in cleaned 10-day embryonic vertebrae are primarily postmitotic. They are small, rounded cells with a high nucleo/cytoplasmic ratio, and the great majority (~98%) are embedded in a metachromatic matrix. Fig. 1 is a radioautograph of an intact vertebra exposed to S^{35}-sulfate for ½ hr. Grains are present above *all* the cells and *all* the matrix. There is a striking uniformity in the numbers of grains over the cells surrounded by matrix. Even after this short pulse, the number of grains over the matrix tends to be higher than the number of grains above the cells. The small number of cells at the periphery not surrounded by matrix exhibit a lower number of grains. If vertebrae exposed to S^{35}-sulfate for ½ hr are then grown in cold medium for 4 days, the matrix remains heavily labeled though the number of grains above the cells decreases (Pelc, 1955; Dziewiatkowski, 1962).

There is no incorporation of S^{35}-sulfate by vertebrae which have previously been frozen and thawed. Any S^{35}-sulfate incorporation by the vertebrae, therefore, is a result of active incorporation and not of nonspecific binding of the isotope.

Fig. 2 is an autoradiograph of a vertebra exposed to H^3-proline for 24 hr. Again, grains are observed above *all* the cells and *all* the matrix. However, the number of grains above the cells is considerably higher than the number above the matrix. Fig. 4 is a section of a vertebra grown in cold medium for 3 days after exposure to H^3-proline. The number of grains over the matrix is still high, but the number of grains over the cells has diminished considerably. The clustering of grains over all the cells after a pulse and the clustering over the matrix 3 days after the pulse is consistent with the notion that all the chondrocytes

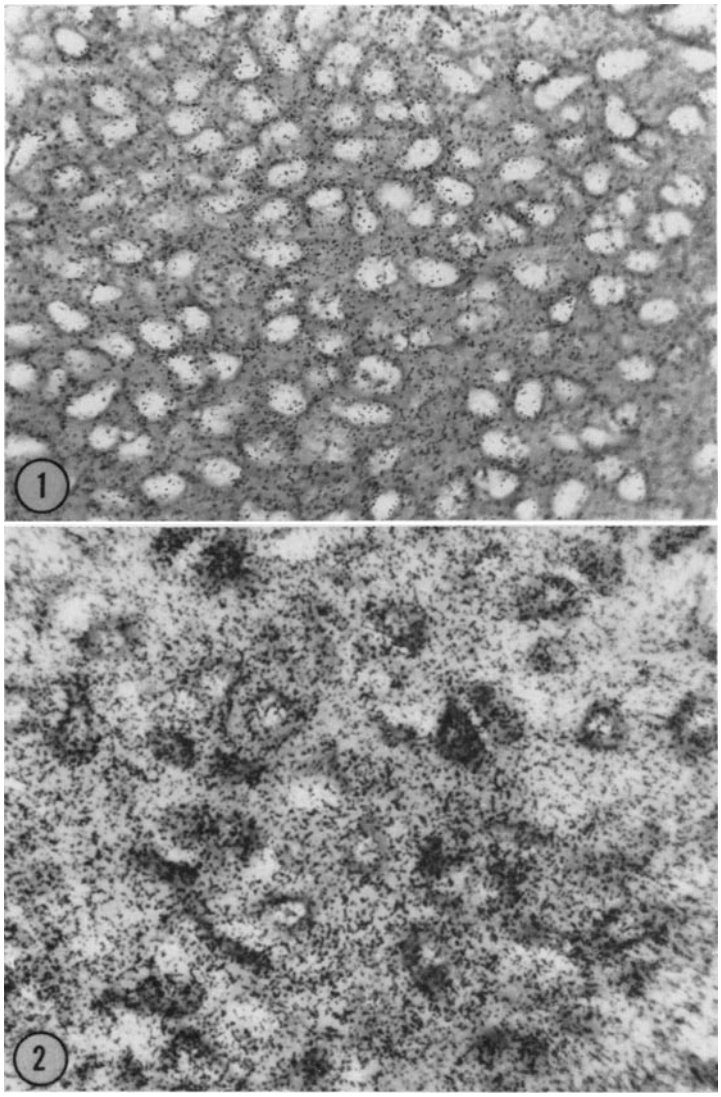

FIGURE 1 Radioautograph of a 10-day chick embryonic vertebra (4-μ section) in organ culture 24 hr, exposed to S^{35}-sulfate for ½ hr, and stained with toluidine blue. Every cell is surrounded by extracellular, metachromatic matrix which appears dark in the photomicrograph. All the chondrocytes incorporate the isotope to approximately the same degree. × 250.

FIGURE 2 Radioautograph of 10-day embryonic vertebra in organ culture 24 hr, exposed to H^3-proline for 24 hr, and stained with toluidine blue. Grains are present above all the cells and matrix. There are more grains above the cells than above the matrix. × 450.

are secreting collagen. An earlier biochemical analysis of this material demonstrated that much of the isotope is hydroxylated by the chondrocytes, forming hydroxyproline which, in turn, is found in the collagen fraction (Prockop, Pettengill, and Holtzer, 1964).

Fig. 3 is a radioautograph of an intact vertebra exposed to H^3-thymidine for 4 hr. Very few encapsulated cells (less than 1 in 1,000) show grains over their nuclei. Most cells which have incorporated H^3-thymidine are located on the periphery and are not surrounded by matrix.

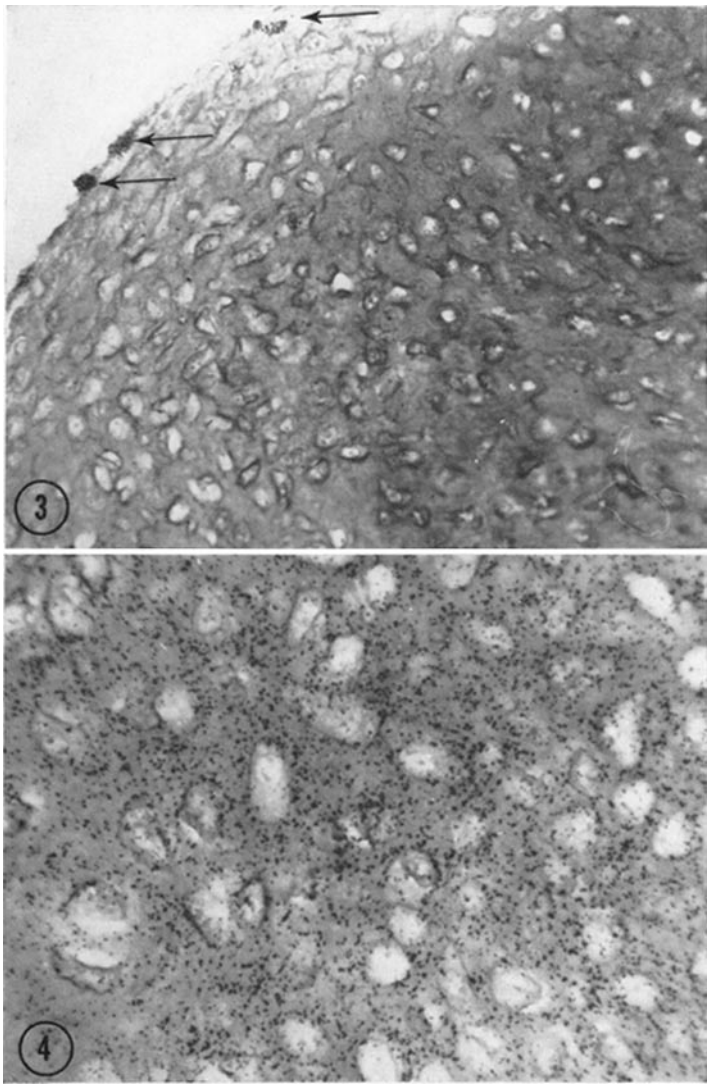

FIGURE 3 Same material as in previous figures but exposed to H^3-thymidine for the last 4 hr before sacrificing. Labeled cells, indicated by 3 arrows, are found on the periphery of the vertebra. The dark-staining extracellular matrix is metachromatic. × 160.

FIGURE 4 A 10-day vertebra exposed to H^3-proline as in Fig. 2, but then grown for an additional 3 days in cold medium. The matrix is evenly labeled. The number of grains above the cells is distinctly lower than the number of grains above the matrix. × 450.

These observations suggest that the vast majority of chondrocytes in 10-day vertebrae are homogeneous both in their cytology and in their commitment to the concurrent production of sulfated polysaccharide and collagen. By definition, these cells are genetically determined to synthesize these specific molecules. Most of these cells are not synthesizing DNA. The very small number that are synthesizing DNA are not surrounded by substantial amounts of metachromatic matrix.

Characteristics of Liberated Chondrocytes Grown in Organ Culture

Immediately after chondrocytes are liberated from their matrix by digestion with trypsin, they

appear as small rounded cells devoid of detectable metachromatic matrix. When these cells (1 to 5 × 10^6) are centrifuged into a pellet and organ-cultured in 2:2:1, many become surrounded by newly made metachromatic matrix within 5 hr. After 5 days in culture, over 98% of the cells are surrounded by metachromatic capsules. When 3- to 5-day-old pellets are exposed to S^{35}-sulfate for ½ hr and analyzed radioautographically, grains are observed over cells and matrix, though more are over the matrix than over the cells. Pellets exposed to H^3-proline for 24 hr exhibit grains primarily over the cells and relatively few over the metachromatic matrix. If, after exposure to S^{35}-sulfate or H^3-proline, the pellets are reared in cold medium for 3 days, the grains persist above the matrix, but diminish above the cells.

Pelleted chondrocytes exposed for 4 hr to H^3-thymidine after 18, 64, 114, and 159 hr of culture and analyzed radioautographically show a maximum of 3 % of the cells incorporating the isotope (Fig. 12). As was found in the intact vertebrae, the majority of labeled cells are peripherally located and not embedded in matrix (Fig. 5).

These results demonstrate that the cytology and synthetic behavior of the centrifuged chondrocytes cultured in pellets are indistinguishable from those of chondrocytes in intact vertebrae. The liberation procedure per se does not grossly injure the synthetic machinery of the chondrocytes. The observation that over 98% of the cells become surrounded by metachromatic matrix indicates that the initial cell population consisted almost exclusively of chondrocytes. Thus, the behavior of these cells to be described in the following sections cannot be attributed to selection between different kinds of cells.

Cytological Changes of Chondrocytes Grown in Monodisperse Cultures for 48 Hr

Aliquots of 2 × 10^5 cells were plated out in 2:2:1 on top of, or in, a thin plasma clot. By 15 hr in culture, over 75% of the cells change from a rounded to a flattened, stellate shape. These flat cells do not exhibit any recognizable cartilage matrix when stained with toluidine blue. By 48 hr, virtually all cells are stellate and show no metachromasia (Table I and Fig. 6).

A minority of the cells remain rounded at 15 to 20 hr, and they are encased in metachromatic capsules (Figs. 7 and 9). Even such cells, however, will abandon their capsules within a few hours if they do not contact other chondrocytes. The cells appear to escape by crawling through breaks in the capsule, leaving behind relatively intact meta-

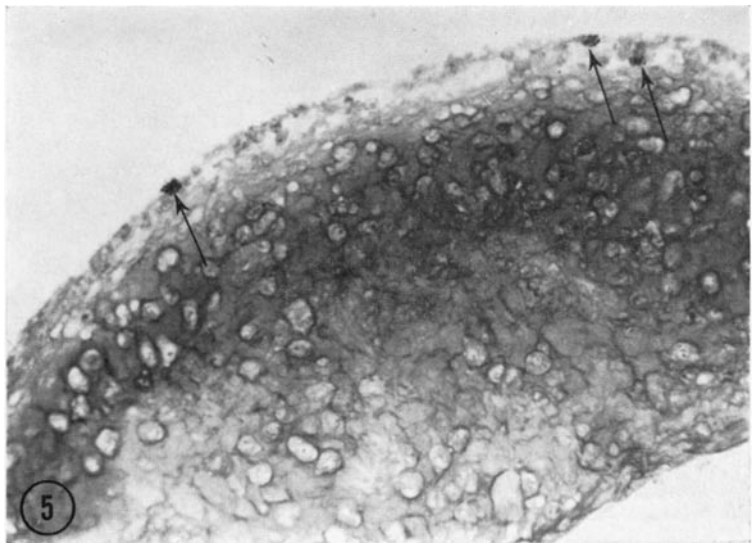

FIGURE 5 Radioautograph of a pellet (4-μ section) made from liberated chondrocytes, in organ culture 3 days, exposed to H^3-thymidine for the last 4 hr before sacrificing. Stained with toluidine blue. Most cells are surrounded by metachromatic matrix which appears dark in the photomicrograph. Labeled cells, indicated by the 3 arrows, are located on the periphery of the pellet. Compare with Fig. 3. × 160.

TABLE I

Cell Morphology and S^{35}-Sulfate Incorporation by Chondrocytes in Vitro

Liberated chondrocytes in monodisperse cultures were continuously exposed to S^{35}-sulfate for various periods of time. The number of grains/100 round cells was counted. Grains over cells in clusters were too dense to count accurately so the quantity is indicated by +++. "Ghosts" refer to the shed metachromatic capsules.

Time in culture	Rounded cells	Stellate cells	Metaphase plates	Grains/100 rounded cells	Grains/100 stellate cells	Grains/100 cells in clusters
hr	%	%	%			
1	100	0	0.01	80 (0–8)	—	—
5	95	5	0.01	978 (0–42)	122 (0–7)	1251
15	22	78	0.01	4507 (16–82)	594 (0–21)	+++
24	7	93	0.05	4022 (13–76)	828 (0–28)	+++
48	2	98	6.0	Ghosts	535	++

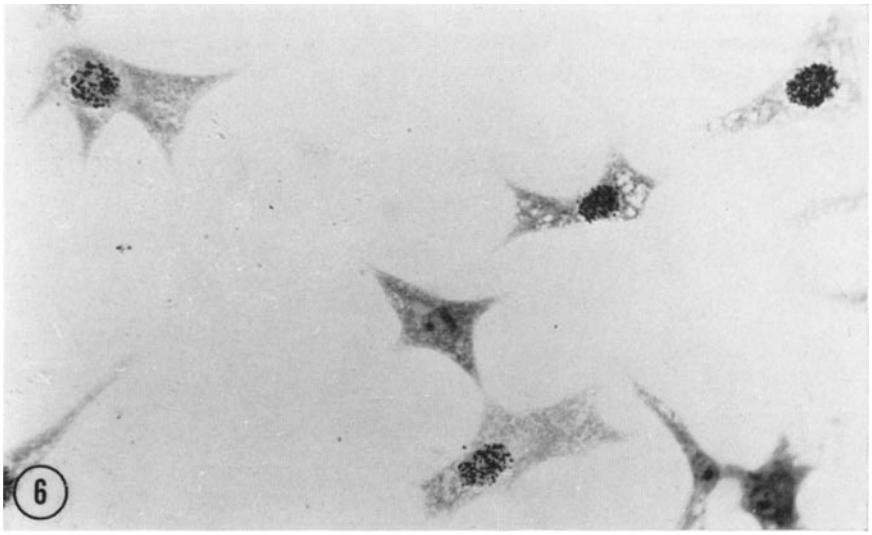

FIGURE 6 Radioautograph of low density cultures of liberated chondrocytes (3×10^5) exposed to H^3-thymidine between 18 and 22 hr. Many of the stellate cells are labeled. There are no round cells in this microscopic field, and there is no metachromatic material associated with the stellate cells. × 450.

chromatic "ghosts" (Figs. 7 and 8). Many vacated capsules are found in 18- to 24-hr cultures. Occasionally, capsules with pseudopodial projections are seen, suggesting that the polysaccharide shell is distensible. Only rarely do cells in the monodisperse condition retain their capsules in 2:2:1 for more than 48 hr.

Groups of two or three cells have more metachromatic material between their apposed surfaces than on their free surfaces. Cells in clusters

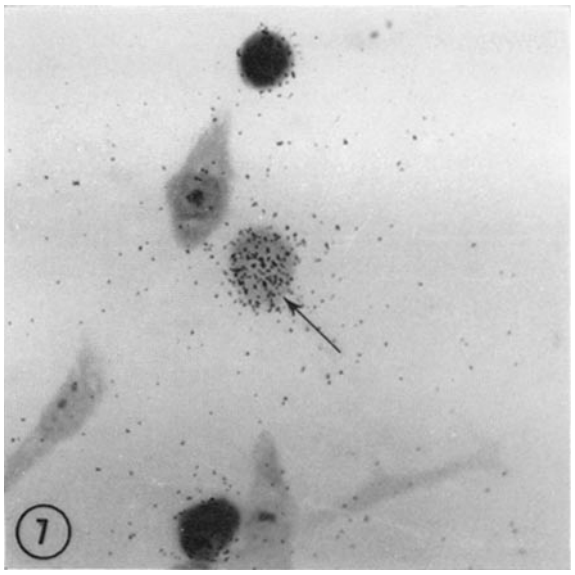

FIGURE 7 Cultures of liberated chondrocytes were exposed to S^{35}-sulfate from 0 to 12 hr and subsequently grown in nonradioactive medium. A labeled metachromatic "ghost" is seen in a radioautograph of a 20-hr culture (arrow). There are many grains above the two round, intensely metachromatic cells (i.e., the dark cells), but, owing to the level of focus and length of exposure, the individual grains are not resolved. Pycnotic nuclei have never been observed associated with the "ghosts." × 450.

retain their capsules longer than do isolated encapsulated cells. Eventually the cells in small clusters spread and lose their capsules. Clearly the presence of matrix deposited in vitro around isolated cells, or around small clusters, is not sufficient in itself, under these in vitro conditions, to maintain them as cytologically recognizable chondrocytes. The difference in behavior of equivalent chondrocytes grown in monodisperse cultures and those grown in pellets, with regard to the continued formation of polysaccharide matrix, is very striking. These results confirm and extend previous observations of chondrocytes in vitro (Fischer, 1922; Holtzer, 1961; Stockdale. Abbott, Holtzer, and Holtzer, 1963; Kuroda, 1964 *a* and *b*).

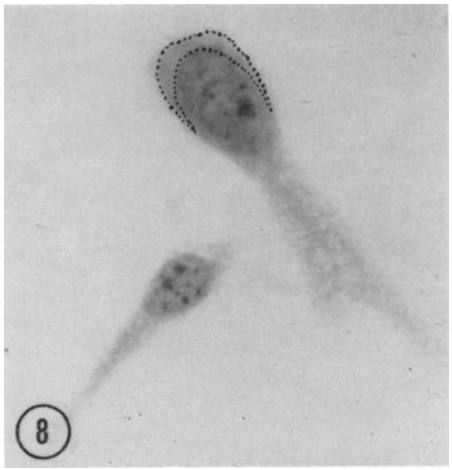

FIGURE 8 Chondrocytes 20 hr in culture, one of which is in the process of shedding its metachromatic capsule. The metachromatic capsule is outlined by dotted lines. The cytoplasm extends through a break in capsule and is spread out on substrate. The nucleus is partially inside the capsule. This cell represents one of many stages observed as the cells abandon their capsules. × 900.

Radioautography of S^{35}-Sulfate Incorporation in Monodispersed Chondrocytes

The transformation of chondrocytes from a rounded to a stellate condition accompanied by failure to persist in matrix deposition led to the hypothesis that cell shape might be correlated with the synthesis of chondroitin sulfate. To test this hypothesis, monodisperse cultures of 2×10^5 chondrocytes grown in 2:2:1 were exposed *continuously* to S^{35}-sulfate for 1, 5, 15, 24, and 48 hr and the radioautographs were analyzed for grains over rounded *versus* stellate cells. From Fig. 9 and Table I it is evident that rounded cells incorporate more sulfate than do stellate cells and that the most sulfate per cell is incorporated by cells in clusters. There is no further increase in total S^{35}-sulfate incorporation into the rounded cell population after 15 hr. If there is no turnover, and if the encapsulated cells continue to take up the isotope after 15 hr, the counts over individual rounded cells should be higher at 24 hr than at 15 hr. 50 fields were scanned in both the 0- to 15-hr and the

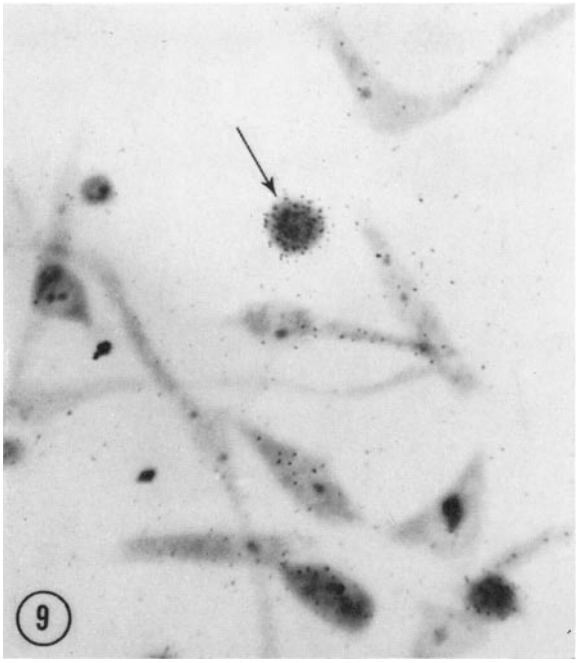

FIGURE 9 Radioautograph of S^{35}-sulfate incorporation by liberated chondrocytes (3×10^5) grown in monodisperse culture. The culture was exposed continuously to S^{35}-sulfate for 15 hr, radioautographed, and stained with toluidine blue. There are many grains over the encapsulated round cell (arrow) and relatively few over the stellate cells. × 450.

0-to 24-hr series, and the 20 most heavily labeled cells were chosen to be counted. On an average, there were 87 grains/cell in the 0-to 15-hr series and 73 grains/cell in the 0- to 24-hr series. Either these cells incorporate S^{35}-sulfate for a maximum of 15 hr or, after working for 15 hr, achieve a steady-state level in the turnover of bound isotope. Thereafter, even if capsules are present, the cells do not accumulate additional molecules of sulfated polysaccharide.

The earliest observable capsular material appears after 5 hr in culture and is always labeled. This is evidence that most of the capsules secreted in vitro are the result of active synthesis in vitro rather than of secondary release of stored chondroitin sulfate previously synthesized in vivo. The absence of unlabeled metachromatic capsules indicates that chondroitin sulfate is not stored in large quantities in the liberated chondrocytes.

Cultures of chondrocytes (2×10^5) were exposed to 1-hr *pulses* of S^{35}-sulfate at 10, 18, 32, and 42 hr. Radioautographs were analyzed for the numbers of grains over stellate and rounded cells (Fig. 11). The level of incorporation by stellate cells never reaches the maximum incorporation level of the rounded cells at 10- and 18-hr. The rate of incorporation by the rounded encapsulated cell population decreases after 18 hr, and by 33 to 43 hr has reached levels of incorporation approximating those of the stellate population. The state of roundness in itself, then, under these in vitro conditions is not sufficient for isolated cells to maintain the synthesis of chondroitin sulfate. Furthermore, the presence of a capsule around a chondrocyte does not necessarily mean that the cell is actively synthesizing chondroitin sulfate at all times.

At no time were metachromatic "ghosts" observed to have incorporated the isotope. Apparently, the cell must be present for the polysaccharide to be sulfated.

When cultures of 2×10^5 liberated chondrocytes were exposed to S^{35}-sulfate from 0 to 12 hr and subsequently reared in nonradioactive medium, many labeled "ghosts" appear after 20 to 36 hr (Fig. 7). The "ghosts" retain both their structural integrity and their sulfate label, suggesting that little degradation of the labeled polysaccharide occurs in the shedding process. By 48 hr, the number of "ghosts" has declined considerably, and by 72 hr they can no longer be observed.

It is worth stressing that S^{35}-sulfate is found above stellate cells. The amount of S^{35}-sulfate incorporated into stellate cells is comparable to that incorporated into embryonic liver, kidney, muscle, skin, spinal cord, dedifferentiated cartilage cells (unpublished results) as well as into fibroblasts in vitro (Mancini, 1956). Clearly a

relatively modest uptake of S^{35}-sulfate cannot by itself be taken as proof of the synthesis of chondroitin sulfate.

To summarize: CSA (chondroitin sulfate) synthesis is shut off within hours in the majority of chondrocytes grown in a monodispersed condition. The reduction in the incorporation of S^{35}-sulfate is associated with a change from a rounded to a flat shape and an absence of metachromatic material. Single chondrocytes which have synthesized new capsular material are unable, as isolated cells under these culture conditions, to persist in this activity.

DNA Synthesis and Mitotic Activity of Liberated Chondrocytes in Vitro

Low-density cultures of chondrocytes (2×10^5) were analyzed for numbers of metaphase plates at 1, 5, 15, 24, and 48 hr (Table I). The per cent of metaphase plates rises from 0.01 at 15 hr to 6.0 at 48 hr. The great majority of metaphase plates are found in nonencapsulated cells.

Low-density cultures of liberated chondrocytes (2×10^5) were grown in 2:2:1, exposed for 4 hr to H^3-thymidine beginning at 12, 16, 18, 20, 64, 114, and 159 hr and analyzed radioautographically for numbers of cells having grains over their nuclei (Figs. 6, 10, 12). The per cent of cells synthesizing DNA rises abruptly on the 1st day of culture. The highest per cent (26.8) of cells incorporating H^3-thymidine occurs 18 to 22 hr after plating. At this time, most of the cells have ceased incorporating S^{25}-sulfate and few metachromatic encapsulated cells are present. The fluctuations in the per cent of cells incorporating H^3-thymidine may be attributable to a partial synchrony of DNA synthesis in these cultures. The low-density cultures were inspected for (*a*) H^3-thymidine–labeled cells with no capsules and (*b*) H^3-thymidine–labeled cells with capsules. Table II shows that at no time are more than 2% of the encapsulated population incorporating H^3-thymidine. The per cent of nonencapsulated cells synthesizing DNA in 4 hr rises from 3.7% at 12 to 16 hr to 26.8% at 18 to 22 hr.

If low-density cultures are grown continuously in H^3-thymidine from 0 to 36 hr, over 90 % of the nuclei are labeled. This rapid rate of cell multiplication continues well into the 2nd day of culture. A more detailed analysis of the synthesis of DNA and cell multiplication in these cultures will appear elsewhere (Bryan and Holtzer, in press).

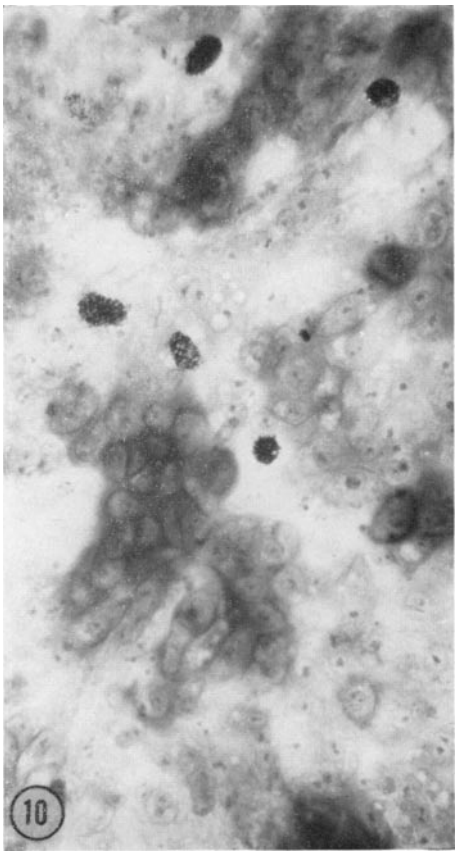

FIGURE 10 Radioautograph of a high density culture of liberated chondrocytes (3×10^6) exposed to H^3-thymidine between 18 and 22 hr. The labeled cells are *not* in capsules. Note the appreciable amount of metachromatic matrix that has already formed in these high density cultures. × 450.

High-density cultures (3×10^6)—a condition between pellets and low-density cultures—were exposed to H^3-thymidine according to the same schedule used for the pellets (Fig. 12). An initial burst of DNA synthesis occurs, followed by a drop in the numbers of labeled cells to the level found in pellets and in intact vertebrae. Many clusters of encapsulated cells are found in these cultures (Fig. 10). H^3-thymidine is found primarily in nonencapsulated cells. The encapsulated cells are round and in all respects indistinguishable from the chondrocytes in vertebrae and pellets.

A study of the numbers of mitotic figures was made in the high-density cultures (Table III). The liberated chondrocytes were grown in either 2:2:1 or MEM plus horse serum and analyzed on

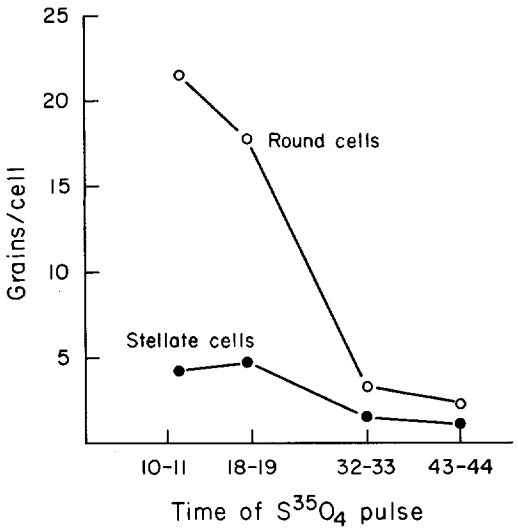

FIGURE 11 A comparison of the number of grains produced by S^{35}-sulfate above round cells as compared with that above the stellate cells.

TABLE II

DNA Synthesis in Encapsulated vs. Nonencapsulated

Time in H^3-Thymidine	Labeled cells/ Encapsulated cells		Labeled cells/ Nonencapsulated cells	
hr	*counted*	%	*counted*	%
0–12	9/334	2.6	113/1726	6.5
12–16	6/413	1.4	50/1347	3.7
16–20	8/432	1.8	278/2346	11.8
18–22			278/1041	26.8
20–24	4/400	1.1	393/1566	25.0

days 3 and 6. There are many more mitotic figures observed in nonencapsulated cells than in encapsulated cells. Neither the length of time in culture nor the kind of medium used has any detectable effect on this correlation. The actual number of mitoses in encapsulated cells is most likely lower than the values show, since a mitotic figure in a stellate cell lying on top of a cluster of encapsulated chondrocytes may be erroneously counted as being surrounded by a capsule.

To summarize: DNA synthesis is induced in chondrocytes liberated from their matrix and grown in monodispersed cultures. The majority of cells which synthesize DNA and divide are not encapsulated, regardless of the culture situation. With increased cell density, the numbers of cells synthesizing DNA decrease.

The Effect of Different Media on the Behavior of Chondrocytes in Vitro

The behavior of cells in tissue culture varies in different kinds of media (Stewart and Kirk, 1954; Eagle and Piez, 1962; Okazaki and Holtzer, 1965). Chondrocytes grown in 2:2:1 or in MEM plus horse serum behave in characteristically different ways. For example, within 48 hr all the liberated chondrocytes (2×10^5) grown in 2:2:1 transform into stellate cells and no metachromatic capsules are found. In contrast, when the same number of chondrocytes are grown in MEM plus horse serum for 48 hr, the metachromatic capsules formed in vitro are retained. There are fewer encapsulated cells after a week, but not until 18 to 20 days do all the capsules disappear from the cultures. In cultures of chondrocytes grown in 2:2:1, on the other hand, encapsulated cells reappear at about 10 days and continue to increase in number with time.

These experiments demonstrate that the behavior of genetically determined chondrocytes is readily influenced by "trivial" changes in the ambient medium.

Relationship of Cell Density to Chondrogenesis

Chondrocytes in clusters incorporate more S^{35}-sulfate per cell and retain their capsules for a longer time in culture than do isolated encapsulated cells. The following experiments were designed to test the proposition that whether or not the "genetically" -determined chondrocytes continue to synthesize chondroitin sulfate depends upon their close association with other chondrocytes.

Clots made on coverslips tend to be convex. Clots made directly on the bottom of Leighton tubes tend to be concave. When liberated chondrocytes are added (2×10^5–5×10^6 cells per culture in 2:2:1 or MEM plus horse serum) to these respective clots, a gradient in the density of cells is established. On the convex clots, more cells settle along the edges. On the concave clots, more cells settle in the center. In the series with convex clots, the majority of chondrocytes embedded in matrix are found along the edges. In the series with concave clots, the majority of chondrocytes in matrix are found in a strip down the center. Apparently, a certain density of chondrocytes is optimal for keeping the cells committed to the synthesis of chondroitin sulfate.

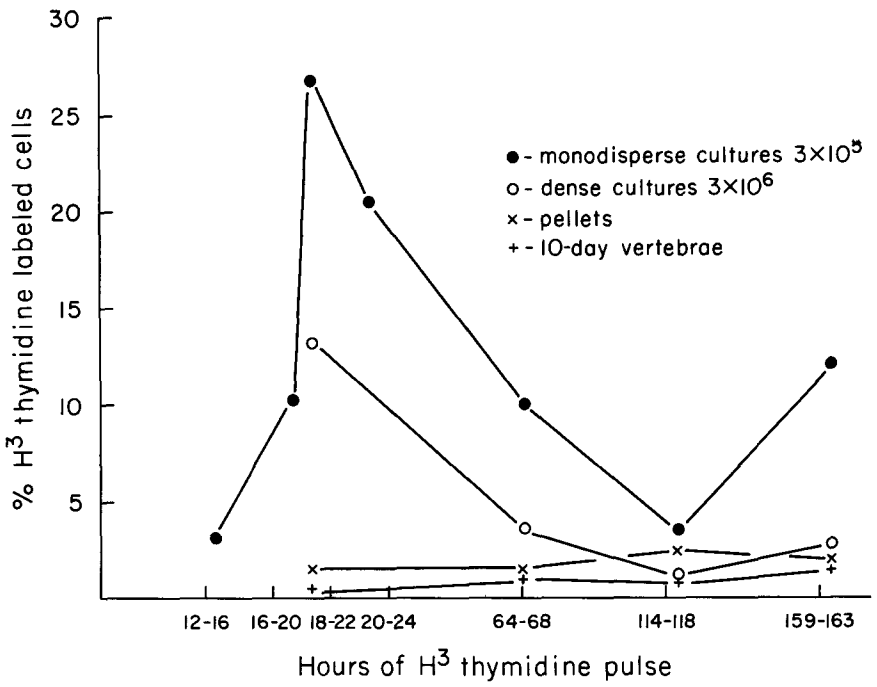

FIGURE 12 A comparison of the numbers of cells incorporating H^3-thymidine when the cells were grown in: (●) monodispersed, low density cultures, (O) confluent, high density cultures, (x) pellets, and (+) intact vertebrae.

TABLE III

Mitotic Activity of Chondrocytes in Vitro

Days in culture	Medium	Stain*	Mitotic figs./Stellate cells		Mitotic figs./Encapsulated cells	
			counted	%	*counted*	%
3	Eagle's	AB	37/1080	4.8	4/698	0.6
		TB	31/900	3.4	1/829	0.1
	2:2:1	AB	48/1107	4.1	6/1191	0.5
		TB	68/3000	2.3	3/978	0.3
6	Eagle's	AB	17/1107	1.5	1/1013	0.1
		TB	7/1025	0.6	0/941	0.0
	2:2:1	AB	42/1080	3.8	1/1426	0.1
		TB	105/2150	4.4	2/1560	0.1

* AB refers to material stained with alcian blue; TB to material stained with toluidine blue.

TABLE IV

Clustering of Chondrocytes in Vitro

No. of cells	Days in culture	Isolated encapsulated cells	Clustered encapsulated cells	Clustered encapsulated cells
		counted	*counted*	%
5×10^4	10	13	378	96
3×10^5	2	25	374	93
	3	16	353	95
	5	14	100	87
1×10^6	3	39	383	91
	6	14	381	96
	10	26	359	93
	Total	147	2328	94

* A cluster is defined as two or more contiguous cells.

Chondrocytes were grown in MEM plus horse serum in three cell concentrations (5×10^4, 3×10^5, 1×10^6), sacrificed at intervals between day 2 and day 10, and stained with toluidine blue. Counts were made of the numbers of *isolated* encapsulated cells *versus* the numbers of *clustered* encapsulated cells. Table IV shows that the majority of encapsulated cells (average, 94%) are found clustered with other chondrocytes irrespective of the initial cell concentration or the length of time in culture.

As described in previous sections, low concentrations of chondrocytes (2×10^5) grown in 2:2:1 change their shape from rounded to stellate, begin to multiply, and are no longer associated with metachromatic material. These cells continue to multiply and after 7 days, in areas of highest cell density, become rounded and metachromatic capsules are observed. As in the shorter term cultures, neither presence of mitotic figures nor incorporation of H^3-thymidine was observed in the encapsulated cell population. With time, increasing numbers of cells are recruited to synthesize chondroitin sulfate. By 20 days, owing to multiplication and subsequent transformation into chondroitin sulfate–synthesizing cells, a multilayered cartilaginous sheet is formed.

These experiments unequivocally demonstrate that the stellate progeny of rounded cells which have stopped synthesizing chondroitin sulfate can, under certain conditions, transform back into chondroitin sulfate–synthesizing cells. This transformation is accompanied by a reacquisition of the rounded shape and a loss of DNA synthesis.

DISCUSSION

One of the main findings of this investigation is the rapid promotion of DNA synthesis in chondrocytes which, if left in their normal environment, will not divide but will continue synthesizing chondroitin sulfate and collagen. By 36 hr, cells in monodispersed cultures no longer incorporate S^{35}-sulfate into detectable matrix, whereas over 90% of the cells will have incorporated thymidine into their DNA (see also Okazaki and Holtzer, 1965). The small amount of DNA synthesis by chondrocytes in pellets demonstrates that their liberation per se does not commit the cells to division. Similarly, it is unlikely that a limiting metabolite in the medium stimulates multiplication, since all cells were grown in the same medium. Rather, the data suggest that the stimulus for division acts on the monodisperse cells after plating and that it is the absence of physical interaction between chondrocytes which promotes DNA synthesis.

The cell surfaces of chondrocytes are confronted with a novel microenvironment when dispersed in vitro. They respond by flattening, adhering to the substrate, and displaying vigorous amoeboid movement. These amoeboid cells cease synthesizing cytologically detectable chondroitin sulfate and begin to divide (see also Willmer and Jacoby, 1936).

If, however, liberated chondrocytes are centrifuged and grown as a pellet, they remain small and rounded and continue to synthesize chondroitin sulfate and collagen. Though it is not demonstrated, we assume that cells in pellets are relatively immobile and that their cell surfaces are not engaged in the extensive amoeboid movement of isolated chondrocytes. The small dividing population in pellets and vertebrae are flattened nonencapsulated cells found on the periphery, a location which allows the cell a maximal surface area and may even allow cell movement. Essentially similar observations have been reported by Wessells (1964) on the relationship between the formation of zymogen granules and the synthesis of DNA by cells in the pancreatic anlagen.

It is proposed that a chondrocyte whose cell membrane is engaged in amoeboid movement cannot make chondroitin sulfate. It is believed that adherent chondrocytes reciprocally stabilize their cell membranes, a process which, in some manner,

allows them to continue to make chondroitin sulfate. This theory and the concept of contact inhibition proposed by Abercrombie and Heaysman (1954) are obviously analogous.

By flattening, the stellate cells change their surface to cytoplasmic ratio, and their effective surface area increases over 6-fold (Holtzer, 1964). Revel and Hay (1963, 1964), Godman and Lane (1964), and Porter (1964) have implicated the Golgi apparatus in the production of chondroitin sulfate and collagen. Robbins and Gonatas (1964) and others report that the Golgi body is poorly developed in rapidly dividing cells in vitro. It would be of interest to know whether the Golgi body in the stellate chondrocytes is poorly formed. This could be the result of the recruitment of intracellular membrane components into the expanded cell surface.

Effective cross-talk between cells which promotes polysaccharide synthesis and restrains DNA synthesis is not dependent upon cell density alone. The reaction occurs between homotypic cells but not between heterotypic cells. Chondrocytes juxtaposed to the surfaces of liver, kidney or muscle cells in monolayer cultures behave as monodisperse cells and do not make chondroitin sulfate, in spite of the contiguity with, or the density of, the heterotypic cells (Abbott and Holtzer, 1964). This interference with chondroitin sulfate synthesis by heterotypic neighboring cells is reciprocal. For example, not only do neighboring myogenic cells interfere with the genetic expression of chondroblasts, but the chondroblasts interfere with the fusion and synthesis of myosin by the myogenic cells.

It is often said that there is an antagonistic relationship between proliferation and differentiation; that specialized cells do not divide and that dividing cells are not specialized. This useful generalization is only a first approximation. For example, hematocytoblasts, crypt cells of the intestine, liver cells, and skin cells are all capable of cell division. Presumably, each of these dividing cells is differentiated. Chondrocytes that would not have divided were induced to do so by altering their environment. Apparently, for the chondrocyte to resume multiplication, it must cease making chondroitin sulfate and collagen.

Observations on embryonic muscle cells (Holtzer, 1961; Stockdale and Holtzer, 1962; Okazaki and Holtzer, 1965) have stressed the mutual exclusivity of DNA synthesis and myosin synthesis. These and related observations on other cell types (Wessells, 1964; Takata, Albright, and Yamada, 1965) suggest that the relationship between cell differentiation and cell division may be more accurately summarized as follows: A cell committed to the synthesis of specialized somatic molecules will not *concurrently* engage in the synthesis of molecules uniquely required for DNA synthesis and cell multiplication. According to this formulation, a liver cell synthesizing albumin or even a normoblast synthesizing hemoglobin, if replicating, would first suppress the synthesis of albumin or hemoglobin, respectively. This might mean that when certain integrated gene sets are transcribing, it is mandatory that others be repressed (Masters and Pardee, 1965). The several recent reports (Goldberg and Green, 1964; Castor and Muirden, 1964) that rapidly dividing fibroblasts do not synthesize collagen in their rapid growth phase, but do when the cell density rises, is consistent with this view of proliferation and differentiation.

Dividing cells surrounded by matrix are common in all very early embryonic (5- to 8-day) cartilages. Experiments are underway to determine whether such cells actively synthesize polysaccharide during all phases of the division cycle (G_1, S, G_2, M), or whether the surrounding matrix is secreted by nondividing neighboring cells.

When the stellate cells in these primary cultures, contact other stellate cells, many of them resume the synthesis of chondroitin sulfate. If, however, the primary cultures are subcultured every 4th day for a period of 2 wk, a procedure which maintains rapid multiplication, the serially cultured stellate cells do not resume the synthesis of their normal amount of chondroitin sulfate and collagen when they contact neighboring cells (Holtzer et al., 1960; Stockdale et al., 1963; Prockop, Pettengill, and Holtzer, 1964). It is not clear why the "memory" of the progeny of chondrocytes for the synthetic activities responsible for multiplication should survive for more generations than the "memory" for the synthetic activities responsible for chondroitin sulfate and collagen.

According to many current schemes (e.g., Jacob and Monod, 1961), the shift in behavior of programmed chondrocytes from collagen- and mucopolysaccharide-synthesizing cells to proliferating cells, and vice-versa, should involve the repression of some genes and the derepression of others. Furthermore, although critical experimental evidence is lacking, it is commonplace to

visualize the sequential events in differentiation as due to turning genes "on" and "off." Clearly, an analysis of the interplay between the controls for DNA synthesis and the controls for synthesis of molecules unique to specialized cell types is important for understanding cell differentiation.

This work was supported by grants from the National Institute of Child Health and Human Development (HD-00189 and 5T1 HD 3003) and the National Foundation. Dr. J. Abbott's present address is the Department of Zoology, Barnard College, Columbia University, New York, New York. Dr. H. Holtzer is the recipient of a research career development award (5-K3-HD-2970) from the National Institutes of Health, United States Public Health Service.

Received for publication 8 November 1965.

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