E. Goldberg (Cold Spring Harbor Laboratory), G. Leidy (Columbia University), and J. Rothman (The Rockefeller University).

[16] Experiment done in collaboration with Dr. R. Krug (Sloan-Kettering Institute for Cancer Research).

[17] Dubos, R. J., *J. Exptl. Med.*, **65**, 873 (1937).

[18] Reich, E., I. H. Goldberg, and M. Rabinowitz, *Nature*, **196**, 743 (1962).

[19] Tomasz, A., unpublished observation.

[20] Nava, G., A. Galis, and S. M. Beiser, *Nature*, **197**, 903 (1963).

CLONAL STABILITY AND PHENOTYPIC EXPRESSION OF CHICK CARTILAGE CELLS IN VITRO*

BY HAYDEN G. COON

DEPARTMENT OF BIOLOGY, BRANDEIS UNIVERSITY, WALTHAM, MASSACHUSETTS

Communicated by Boris Ephrussi, November 26, 1965

The development of new procedures and culture media[1-4] made possible the discovery that several differentiated cell types from chick embryos may maintain their differentiated phenotype in clonal cell culture. Skeletal[1] and cardiac muscle,[5] retinal pigment cells,[6] and cartilage cells[7] have been cloned and subcloned with a basically similar result: these differentiated cellular phenotypes are heritable through many generations in clonal cell culture.

The progressive loss of the expression of tissue-specific cellular phenotypic traits in tissue culture, "dedifferentiation," has emerged as one of the notable generalizations of *in vitro* studies and has occasioned much discussion concerning the stability of the differentiated state.[8-10] Initial observations of clonal stability of several differentiated cell types, including cartilage cells, seemed at variance with this generalization. One object of this paper is to show that these opposite results may not be discordant. These experiments demonstrate that "dedifferentiated" populations of cartilage cells may re-express their differentiated functions under suitable culture conditions, and, in addition, show that differentiated cell function, at least at detectable levels, is not a necessary condition for the heritability of the cartilage cellular phenotype.

Materials and Methods.—White Leghorn eggs were used. All cultures were grown in Falcon plastic tissue culture Petri dishes (#3003), 100 × 20 mm, offering an actual culture surface 85 mm in diameter. Cultures were incubated at 36.5°C in a gas-flow, humidified incubator (5% CO_2 in air, 98% relative humidity).

Dissociation of 8-day limb cartilages: Cartilage rudiments from legs (excluding the femur) of six embryos were dissected away from the soft tissue. Adhering perichondrial material was removed by incubating (24°C) whole rudiments for 10 min in standard dissociation medium [CTC: 10% chicken serum in calcium and magnesium-free Tyrodes solution, 0.1% trypsin (Nutritional Biochemicals, 1:300), and 2.5 mg/ml collagenase (Worthington, CLS)], followed by swirling them in four successive changes of CTC at 5-min intervals. Cells were dissociated from the cleaned rudiments by incubation for 20 min in 5 ml of CTC on a gyrorotatory shaker at 37°C. The rudiments were vigorously pipetted and allowed to settle. The supernatant CTC with the liberated cells was aspirated and fresh CTC was added for a final 20-min incubation. The cells were washed immediately by centrifugation with an equal volume of growth medium and were resuspended in

fresh growth medium. The pooled suspensions from both incubations were filtered through two layers of 20-μ nylon filter cloth (*Nitex:* Tobler, Ernst & Traber, Inc., N. Y.), counted, diluted, and plated.

Dissociation of 12–15-day sterna: The perichondrial layers of six sterna were peeled away with watchmaker's forceps, leaving clean cartilage structures. These were cut into pieces about 1 mm^3 and incubated on the shaker as above. The enzyme-resistant matrix of the 12–15-day sternum required 3–4 sequential incubations to achieve complete dissociation.

Media: Ham's F10[3] with amino acids and pyruvic acid at twice the specified concentrations was used. A 2X stock solution of F10 nutrients (4X amino acids, pyruvate) was prepared. Final concentrations of fetal calf serum at 5 or 10% (v/v), 1% bovine serum albumin powder (Armour, fraction V), 1.5 gm/liter additional sodium bicarbonate, and 33 units/ml sodium penicillin G were added before diluting to final volume with Hanks balanced salt solution containing embryo extract. Complete medium was sterilized by pressure through a GS Millipore filter and stored at 2–4°C.

Embryo extract (EE_{50}): Preparation of EE_{50} was adapted from the procedure of Konigsberg.[1] Eleven-day chick embryos were rinsed in cold Hanks solution. The *whole* embryos were drained, weighed, and diluted 1:1 (w/v) with Hanks solution containing phenol red (0.02 gm/liter) and homogenized in an Oster mixer for 40 sec at full power. The homogenate was centrifuged at 30,000 $\times$ g for 30 min and the supernatant was incubated at 4°C with 0.25 mg/100 ml hyaluronidase (Nutritional Biochemicals, type III) for 2 hr. (This step may be deleted without changing the results but the digestion greatly facilitates sterile filtration.) The extract was then centrifuged for 85 min at 110,000 $\times$ g. The pellicle and the pellet were discarded leaving a clear, orange liquid: whole EE_{50}.

Fractionation of EE_{50}, H, and L: Whole EE_{50} was separated into two fractions on a column of Sephadex G-25 coarse, bead-form dextran gel (Pharmacia, N. Y.). Approximately 80 ml of whole EE_{50} was applied to a 2.5 $\times$ 80-cm column equilibrated with Hanks solution without phenol red and then eluted with the same. The H (high mol wt, $\geqq$ 5,000), or excluded, fraction was that part of the eluate containing hemoglobin (determined visually or by readings at 410 and 540 mμ). The L (low mol wt, $\leqq$ 5,000), or retarded, fraction started where the H ended and contained those fractions up to and including the first tinges of phenol red in the eluate. The fractions L and H were corrected for dilution by referring them to the volume of the original sample so that a medium designated H-5 (5% H) contained the same concentration of the high molecular weight components from EE_{50} which would have been present in a medium supplemented with 5% (v/v) whole EE_{50}. Control media without any EE_{50} supplement were designated w/o-media.

Passages: The quantitative serial passages were made by incubating rinsed plates at 37°C with CTC (collagenase 0.75 mg/ml for passages). Gentle pipetting of CTC loosened most cells from the plates after 20 min; a total of 30 min incubation yielded quantitative recovery of cells. The cells in CTC were diluted with 5 ml of growth medium (used to rinse the plates), and were pelleted in a clinical centrifuge. The supernatant solution was decanted, and the cells were resuspended in growth medium. The suspensions were diluted, counted in hemocytometers, and then resown at 5×10^5 cells/plate/10 ml ($\simeq$ 88 cells/mm^2) for continued culture and at $\leqq 10^3$ cells/plate/6.5 ml for cloning analysis. Passage plates were not fed. Clonal plates were fed as follows: 3 ml fresh medium was added on the third day, 5 ml on the sixth day, and 5 ml removed before adding 5 ml every third day thereafter. Clonal plates were usually fixed on day 16.

Preparation of plates: After rinsing in Tyrodes solution, the plates were fixed for 5 min in 2.5% glutaric dialdehyde in 50% Tyrodes solution with sodium cacodylate 3.3 gm/liter. After a second rinse, the plates were stained overnight in 2% aqueous toluidine blue. Excess stain was removed by several rinses with absolute ethanol, and the plates were preserved under a thin layer of immersion oil.

Autoradiography of culture plates: Following a 1- or 2-day incubation with isotope in growth medium, the plates were extensively rinsed in carrier-saline, fixed, washed in water, and air dried. Melted liquid emulsion (Kodak, NTB-3) diluted 1:3 with distilled water was poured onto preheated plates in a high relative humidity atmosphere. The emulsion was immediately drained and the plate set up on edge to dry. After 5–14 days' exposure, the plates were developed, fixed, washed, and stained as above.

Criteria for scoring cartilage: Cartilage-making colonies (CMC's) were recognized by (1) the

presence of a metachromatic extracellular capsule, often most abundant toward the center of the colony; (2) autoradiographic demonstration of $S^{35}O_4^{=}$ incorporation; (3) in combination with the above criteria, characteristic cellular and colonial morphology (Fig. 1*A*). Non-CMC's were distinguished by their fibroblastlike growth pattern and their lack of metachromasy or demonstrable sulfate incorporation. Plates were counted at magnifications of 10–30×.

I. *Clonal Stability of Embryonic Chick Cartilage Cells.*—Primary cell suspensions from leg or sternal cartilages yield colonies with clonal plating efficiencies of 50–90 per cent in medium L-7. Depending upon the effectiveness of the procedures for cleaning away perichondrial material, 98–99.9 per cent of these colonies incorporate inorganic sulfate and make metachromatic extracellular matrix, which was identified in previous experiments[7] as containing chondroitin sulfate. Autoradiographs of

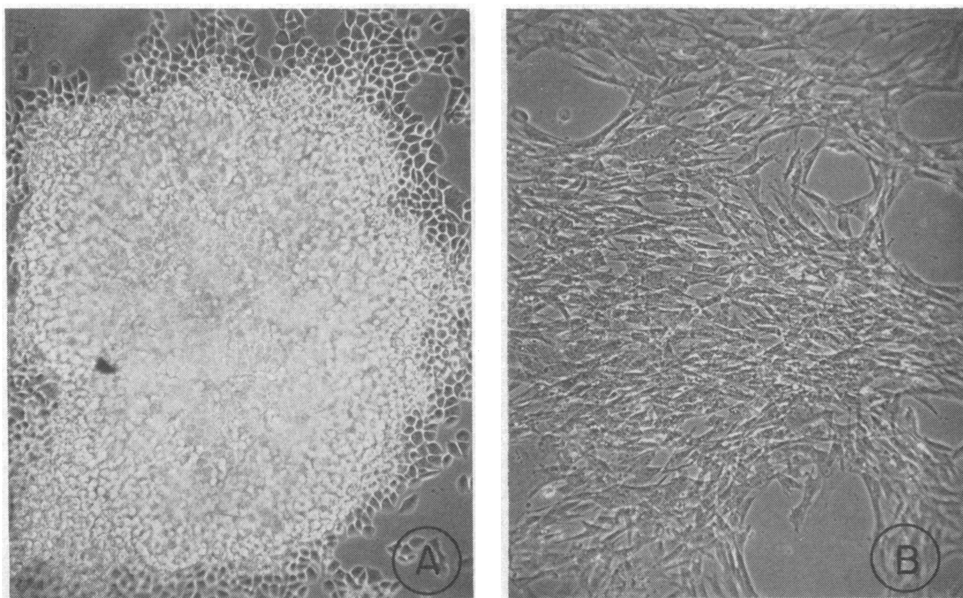

FIG. 1.—(*A*) A phase-contrast photomicrograph of a living cartilage-making colony (CMC) grown in medium L-7 for 18 days. The cells have piled up at the center of the colony and are embedded in a metachromatic matrix. Spread cells at the periphery commonly show wisps of metachromatic material around them. (*B*) A portion of the edge of a sibling clone of that shown in (*A*) but grown for 18 days in medium H-7. Note the typical (non-CMC) fibroblastlike, spreading growth habit. Cells at the center of these colonies typically showed piling up but did not exhibit CMC morphology or function. Both photographs are printed at the same magnification.

clones of 2–8 cells up to several thousand have shown incorporation of sulfate preferentially located over metachromatic material. While the use of cloning cylinders[1, 2] as well as observations of marked cells have verified that true cloning occurs in our cultures, all of the clones counted in this study are statistical clones, i.e., colonies grown from dilute, single cell platings. In these dilute platings, the probability (calculated from the counted frequency of adhering cells) of a single colony being a true clone is $\geqq 0.96$.

Both the CMC's and the fibroblastlike non-CMC's obtained from fresh suspensions of cartilage cells may be subcloned with plating efficiencies between 80 and 100 per cent. Both types of colonies yield subclones like the parent colony. When a colony exhibiting CMC morphology (Fig. 1*A*) is subcloned, the colonies thus

derived also exhibit CMC morphology and, after fixation and staining, are determined to possess the CMC phenotype. The distinctive cell morphology of CMC's appears to be very reliable; among thousands of colonies of 200 cells or more examined, all cases have proved to have CMC morphology associated with metachromatic matrix. Non-CMC's have not been observed to initiate production of metachromotic matrix upon subculture in any medium, including conditioned media.[1, 4, 7]

Three CMC's from leg cartilage primary clonal platings and six CMC's from sternum clonal platings have been passed through four successive clonings with retention of CMC function. At each passage the colonies were allowed to grow to about 1000 cells each, and two or three colonies were individually subcloned for each strain. The remaining 200–400 colonies, the sibling clones for each strain, were fixed and stained. At each passage, all colonies of 200 cells or more were found to be CMC's. In later clonal passages, "giant" cells may appear within CMC's as well as scattered throughout mass culture plates. Occasionally, isolated giant cells may possess associated metachromatic material; usually, they do not. Giant cells have not been successfully subcultured. Based upon observation of hundreds of subclonal passages of CMC's, it is concluded that the CMC phenotype is stably heritable under these conditions of clonal cell culture. The question of whether L-containing media might contain "inductor" substances similar to those described by Lash *et al.*[21] is being investigated. There are, however, two kinds of evidence which argue against that interpretation: (1) L-containing media do not yield higher percentages of CMC's from mixed cell populations than do w/o-media;[4] (2) CMC percentages do not change with doses of L varied from 0.1 to 12 per cent. L-containing media have been adopted for these studies because they yield equal or higher plating efficiencies than other media and support twice the growth rates obtained in w/o-media.[4]

II. *Clonal Assay of Rapidly Passed Populations of Cartilage Cells.*—In order to investigate the differences between clonal cultures and rapidly passed, sparsely sown populations, the conditions described by Holtzer *et al.*[11] were duplicated as closely as possible, using cloning medium L-7. Cell suspensions freshly liberated from 8-day leg cartilages and 15-day sternal cartilages were plated at 5×10^5 cells/plate/10 ml L-7. After 2–4 days' growth, prior to reaching confluence, the cells were trypsinized, resuspended, and counted, and then resown at the original density in new passage plates. The passages were repeated in an attempt to produce "dedifferentiated" populations.[11–13] The maximum density reached in the leg cartilage passage plates did not exceed 25×10^5 cells/plate; the sternal cartilage passages did not exceed 35×10^5 cells/plate before passage. At each passage representative plates were fed and cultured for 7–14 days to see whether metachromatic matrix or sulfate incorporation would occur in the mass plates. For leg cartilage passages number 4–13, portions of the cell suspensions from the plates were pelleted and cut into fragments (0.3–2 mm^3) and were grown as organ cultures for 1 or 2 weeks on 0.5 per cent agar in Tyrodes solution, horse serum, and whole EE_{50}, 7:2:1. Clonal assay plates were prepared at each passage in L-7 medium. The population growth curves and the results of the clonal assays for the cartilage phenotype are presented in Figure 2. The striking finding was that even in media which permitted the clonal growth and differentiated function of cartilage cells, *populations* derived from

embryonic cartilages failed to express detectable specialized function. Clonal platings made at each passage, however, showed that even after an average 10^{10}-fold increase in the population, at least 55 per cent of the cells which formed colonies were still capable of re-expressing their differentiated function in readily detectable fashion.

These data do not permit a decision as to whether the occurrence of many (45 %) fibroblastlike colonies in the latest sternal cartilage passages is the result of refractory "dedifferentiation" of chondrocytes, or whether it represents the gradual outgrowth of "contaminant" non-CMC cells detected in clonal platings of the primary suspensions. Clones of non-CMC's grow with generation times of less than 20 hr in L-7, while CMC's have generation times of 24–33 hr in the same plates.

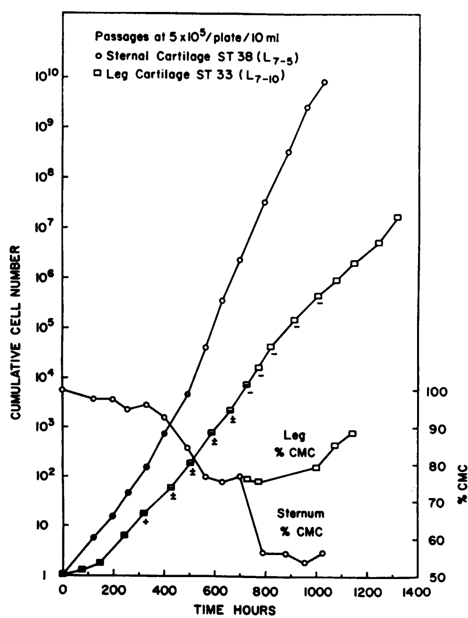

FIG. 2.—Leg cartilage cell suspensions were grown in medium L-7 with 10%, and sternal cartilage with 5% fetal calf serum supplement. Growth curves were constructed by multiplying the number of times the population had increased in each passage by the product of previous passages. The *filled-in symbols* indicate abundant metachromatic matrix occurring on representative passage plates cultured 7–14 days; *partially filled-in symbols* indicate patchy regions of production of metachromatic matrix: *open symbols* indicate no demonstrable matrix formed on the mass plates. Autoradiographs of passage plates incubated with 3 μc $S^{35}O_4^{=}$/ml for 48 hr before fixation matched the results of metachromatic staining precisely. The signs +, ±, −, next to the leg cartilage passages number 4–13, show amounts of metachromatic matrix found in histological preparations of organ-cultured pellets. Superimposed upon the growth curves are the values of per cent (CMC's) counted from clonal platings made at each passage for the sternal cartilage population, and passages number 9, 10, 13–15 for the leg cartilage population. At least 400 colonies were counted at each point. The leg cartilage population was 99.8% CMC at time zero (direct clonal plating); the sternal cartilage population was 99.2% CMC at time zero. Plating efficiency declined 90–100% in the first six sternal cartilage passages to 20% in the final passages; the leg cartilage sample declined from 50 to 20%.

The shift in expression of function of these populations in response to rapid passage is well correlated in both experiments with the attainment of ultimate-slope log phase reproduction. During the lag phase, the *populations* respond to the time-honored procedures[11–14] used for maintaining differentiated function *in vitro.* However, as the populations gradually reach the log phase of multiplication, procedures of massing the cells fail to evince the response typical of earlier passages. and a new phenomenon becomes evident: *dilute* conditions appear to be required for expression of differentiated phenotype in culture.

III. *Response of Lag and Log Phase Cells to Increasing Density.*—These cell populations, while analogous to those studied by others,[11–13] were, like them, not composed of "pure" cell strains; i.e., primary clonal platings revealed 0.2–0.8 per cent fibroblastlike, non-CMC cells present at time zero. It is possible, by pooling

clones, to reduce the probability of initial "contamination" to even lower values. A population made by pooling 50 CMC's from primary culture of 13-day chick sternum was passed five times at 5×10^5 cells/plate/10 ml L-7. At the sixth and seventh passages, after the population had entered log phase, new passage plates were set up at 10^3, 10^5, and 10^6 cells/plate/10 ml L-7. The cultures were continued for 9 days; the medium was changed completely on days 3, 5, and 7. On the ninth day the cultures were fixed and stained with toluidine blue. All of the colonies formed on the 10^3 plates were CMC-positive; many scattered foci of CMC-positive regions dappled the 10^5 plates; but no metachromasy could be found on the 10^6 plates. Freshly isolated chondrocytes, as well as samples from the initial passages of this CMC population, showed metachromatic matrix on all plates up to and including 5×10^6 cells/plate. Attempts to relieve the probable rapid exhaustion of nutrients in critical supply in cultures of high density by feeding plates sown at 10^6 every 6 hr with a complete change of medium did not alter the results.

IV. *Reversible Expression of Cartilage Phenotype in Clonal Cultures.*—Clonal plating made in H-containing media from the rapidly passed populations yielded much lower percentages of CMC's than did samples cloned in L-containing media. Either many CMC's were unrecognizable in H-media, or CMC's were selected against by the EE_{50} H fraction. Fifteen CMC's (1000–1200 cells each) were scraped up from the clonal plates and were individually dissociated in CTC, washed, and resuspended. Half of each colony was sown onto a plate containing 6.5 ml of H-7 medium, and the other half into 6.5 ml of L-7 medium. The plates were fed with the original medium every third day and were fixed and stained on the 16th day of culture. All of the colonies (about 150 for each strain) grown in H-medium showed a fibroblastlike growth pattern (Fig. 1*B*); none of them formed toluidine blue metachromatic matrix. All of the colonies (about 400 for each strain) grown in L-medium were easily identified as CMC-positive by metachromatic staining and their characteristic morphology. Sample CMC-negative colonies of three of the H-grown strains were again subcultured into H-7 and L-7 medium. As before, all the H-grown colonies were CMC-negative, all L-grown colonies were CMC-positive. Control non-CMC's remained CMC-negative throughout identical subculturing.

Dose-response curves (Fig. 3) have been plotted by counting the percentage of colonies which showed metachromatic staining from parallel cultures grown in successively higher concentrations of H. The curves from eight different batches of medium and four different cartilage cell populations have proved to be highly reproducible.

Preliminary attempts to learn more about the reversible CMC-inhibiting substances in the Sephadex (G-25 excluded) H fractions have shown that they are probably large molecules (excluded on Sephadex G-100) and are heat-labile (65°C for 10 min).

The possibility that H-media produce their effect of reversible inhibition of CMC expression by increasing growth rate alone cannot be ruled out completely at present. The average doubling time for clones of cartilage cells in H-5 medium is 21.5 hr; in L-5 or L-7 medium it is about 29 hr. However, preliminary evidence[4] has shown that Sephadex G-100 subfractions of H may support equally high growth rates (21.5 hr), while CMC function is expressed in media containing the subfraction retarded on G-100 and is not expressed in media with the excluded subfraction.

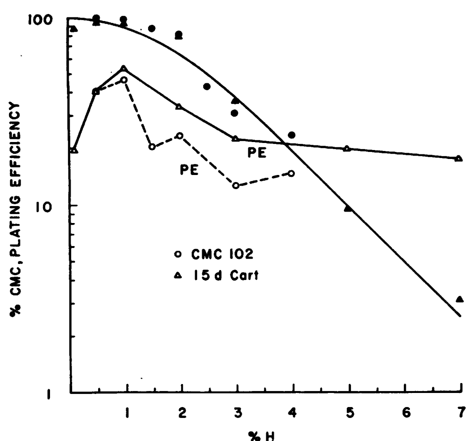

FIG. 3.—A dose-response curve is plotted for two log phase cartilage cell strains in media supplemented with increasing percentages of the H fraction of embryo extract. The *solid triangles* represent the counts of per cent CMC in a population (94.2% CMC in L-7 or w/o media) from 15-day chick sternum. The *solid circles* show the counts for a *clonal* strain, #102 (100% CMC in L-7 and w/o media), derived from a suspension of 13-day chick sternum cells. The curve drawn is a calculated three-hit inactivation curve: $y = (1 - [1 - e^{-x/1.5}]^3)100$. The plating efficiencies for each point are plotted (*open figures*) on the same scale in order to show that the inhibitory effect of H media on CMC expression is not simply related to killing. Each point represents at least 400 colonies counted.

Discussion and Conclusions.—The principal facts to emerge from these experiments are: (1) under clonal (dilute) culture conditions the phenotype of embryonic chick cartilage cells is stable for at least 35 cell generations during four successive clonal passages; (2) populations of cartilage cells have failed to exhibit detectable levels of differentiated function after entering the log phase of growth and, surprisingly, the best method for returning these cells to detectable function is to grow them in dilute culture; (3) it is possible, either in mass culture (inoculation densities $\geqq 5 \times 10^5$ cells/plate), or in the presence of unknown high molecular weight components of EE_{50} (at all inoculation densities tested), for cartilage cells to multiply for at least 20 cell generations (two clonal passages grown to about 1000 cells each) in the absence of detectable function and yet return to express their original differentiated phenotype in permissive media (L or w/o).

These results can be reconciled with those of Holtzer *et al.*,[11] Stockdale *et al.*,[12] and Kuroda.[13] The observed fact of "dedifferentiation" under conditions of rapid cell division in sparsely inoculated cultures has been confirmed with media which otherwise permit clonal growth and retention of differentiated function. This observation has been extended to include evidence that many of the cells in such "dedifferentiated" populations may return to expression of function.

Three of the phenomena discussed here may have influenced the results of earlier observations of "dedifferentiation" in cell cultures: (1) the sensitivity of log phase cells to crowded culture conditions; (2) the effect of inhibitory (H-like) substances from EE_{50}, (3) the possibly inhibitory effects of "contaminant" cell types: their overgrowth may account for the loss of tissue specific function[15] or may inhibit function more indirectly as described recently by Moscona and Moscona.[16]

The finding of Schwartz, Scaletta, and Ephrussi (see ref. 17) that mass cultures of mouse cell strains which fail to produce an esterase (J^-) yield clones which do produce the esterase (J^+) may be similar to the finding that expression of CMC function returns from CMC-negative mass cultures consequent upon cloning. It is not known how generally these conditions permissive to expression may apply to other differentiated cell functions; however, Cahn[4, 6] has recently found that chick retinal pigment cells respond to cloning, crowded culture, and H-media in a manner analogous to cartilage cells.

The existence of a delitescent or latent state of the differentiated cell (in which the differentiated phenotype is not readily detectable) has long been suspected in tissue cultures,[18] is known from mass cultures of pigment cells,[6, 19] and probably occurs in regeneration.[20] It remains to be determined whether synthesis of the characteristic products or the machinery for making them is maintained during rapid growth in the "latent" state.

Summary.—Expression of the differentiated phenotype of embryonic chick cartilage cells is influenced by the medium (embryo extract supplement), the cell density, and the growth phase of the cells. It was possible to obtain clonal and mass culture loss of detectable function, like that previously reported for cartilage cells, as well as clonal retention of function, like that known for skeletal muscle and pigment cells. The apparent paradox was resolved: even after many cell generations most of the cells in a "dedifferentiated" population are capable of re-expressing their differentiated phenotype.

The author acknowledges with deep gratitude the encouragement and advice of Prof. Edgar Zwilling, in whose laboratory the original work was performed. Special thanks are due to Miss Geraldine Ingram for her capable technical assistance. The author wishes to thank Marie Coy Cavanagh for making the radioautographs and for developing this very useful technique.

* This research was supported by grant GB-2619 from the National Science Foundation.

[1] Konigsberg, I. R., *Science*, **140**, 1273 (1963).

[2] Puck, T. T., P. I. Marcus, and S. J. Ciecura, *J. Exptl. Med.*, **103**, 273 (1956).

[3] Ham, R. G., *Exptl. Cell Res.*, **29**, 515 (1963).

[4] Coon, H. G., and R. D. Cahn, *Science*, in press.

[5] Cahn, R. D., *J. Cell Biol.*, **23**, 17A (1964) (abstract).

[6] Cahn, R. D., and M. B. Cahn, these PROCEEDINGS, **55**, 106 (1966).

[7] Coon, H. G., *J. Cell Biol.*, **23**, 20A (1964) (abstract).

[8] Trinkaus, J. P., *Am. Naturalist*, **90**, 273 (1956).

[9] Grobstein, C., in *The Cell*, ed. J. Brachet and A. E. Mirsky (New York: Academic Press, 1959), vol. 1, p. 437.

[10] Davidson, E. H., in *Advances in Genetics*, ed. E. W. Caspari and J. M. Thoday (New York: Academic Press, 1964), vol. 12, p. 143.

[11] Holtzer, H., J. Abbott, J. Lash, and S. Holtzer, these PROCEEDINGS, **46**, 1533 (1960).

[12] Stockdale, F. E., J. Abbott, S. Holtzer, and H. Holtzer, *Develop. Biol.*, **7**, 293 (1963).

[13] Kuroda, Y., *Exptl. Cell Res.*, **35**, 326 (1964).

[14] Fell, H. B., and R. Robinson, *Biochem. J.*, **23**, 767 (1929).

[15] Sato, G., L. Zaroff, and S. E. Mills, these PROCEEDINGS, **46**, 963 (1960).

[16] Moscona, M. H., and A. A. Moscona, *Develop. Biol.*, **11**, 402 (1965).

[17] Ephrussi, B., in *Retention of Functional Differentiation in Cultured Cells*, ed. V. Defendi (Philadelphia: The Wistar Institute Press, 1964), Symposium Monograph no. 1, p. 99.

[18] Strangeways, T. S. P., *Tissue Culture in Relation to Growth and Differentiation* (Cambridge, England: W. Heffer & Sons, Ltd., 1924).

[19] Doljanski, L., *Compt. Rend. Soc. Biol.*, **105**, 343 (1930).

[20] Hay, E. D., in *Regeneration*, ed. D. Rudnick (New York: The Ronald Press Co., 1962), p. 177.

[21] Lash, J. W., F. A. Hommes, and F. Zilliken, *Biochim. Biophys. Acta*, **56**, 313 (1962).