tions for 4 days and given 1 long day with supplementary light varying in duration from 12 to 24 hours at intensities (incandescent light) varying from 300 to 10,800 lux. The photoperiod needed to induce formation of flower primordia in 50 percent of the plants was estimated by interpolation from a series of response curves similar to Fig. 1; 20 replicate plants were used per treatment. When the intensity was 10,800 lux, a photoperiod of 13 hours was sufficient for 50-percent induction, but, when the supplementary light was reduced to 300 lux, 23 hours were necessary (Fig. 2).

The effect of temperature on sensitivity to 1 long day was tested by growing plants continuously at 15°, 20°, 25° or 30°C. Plants were sensitive to 1 long day at all temperatures. Flower initials were formed most rapidly at 30°C, but the plants were excessively etiolated and growth was poor. Flower initiation was only 1 day slower at 25°C, and, as growth was more satisfactory, this temperature is probably the most useful for further experimentation.

This strain of *B. campestris* is therefore a useful addition to the short list of long-day plants sensitive to 1 long day. There are no special requirements for germination, which is rapid and uniform. The seedlings respond to a single long day as early as 4 days after sowing, and inflorescence development is visible on dissection after another 6 days. Large quantities of seed (about 5000 per plant) can be easily obtained by selfing plants grown under normal greenhouse conditions with a day length of about 16 hours.

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Differentiation in vitro: Effects of Sephadex Fractions of Chick Embryo Extract

Abstract. *Chicken embryo extract has been fractionated into high- and low-molecular-weight components on Sephadex G-25. Media supplemented with the low-molecular-weight fraction (L) support full differentiation in clones of cartilage and of pigmented retina cells from chicken embryos. Growth rates of such cultures in L-supplemented media are greater than in media without embryo extract, and plating efficiencies are higher than in media with or without whole embryo extract. The high-molecular-weight fraction (H) in low concentrations also stimulates growth and plating efficiency, but inhibits the expression of differentiation.*

Chicken embryo extract (EE) has been used to enhance the growth of cells in tissue and organ culture (*1*). High concentrations of EE have marked positive and negative effects on cellular differentiation (*2–4*). Both Ebeling (*3*) and Doljanski (*4*) showed that the amount of pigment formed in plasma clot organ cultures of iris epithelium was inversely proportional to the concentration of EE in the medium. On the other hand, others have reported differentiation of organ explants in media containing 20 to 30 percent EE (*2*). In general, however, non-neoplastic cells in monolayer culture lost overt signs of differentiation in media containing EE unless the media had been conditioned beforehand (see *5, 6*).

In experiments designed to improve methods for growing differentiated cells in monolayer culture or as clones we found that high amounts of EE lowered plating efficiencies with cartilage and pigmented retina. Deletion of EE from cloning media decreased both the growth rates and the plating efficiencies.

In order to separate deleterious factors in EE from those which stimulate growth and promote high plating efficiences, EE was fractionated on Sephadex G-25. Whole chicken embryos (9- to 11-day-old White Leghorn) were mixed with an equal amount (weight/volume) of Hanks' solution (*7*) containing 0.01 to 0.02 mg of phenol red per milliliter, and then minced in an Oster blender at top speed for 40 to 60 seconds. The material was centrifuged in the cold at 34,000 *g* for 30 minutes; the supernatant was then decanted and stirred for 1 hour at 0° to 2°C or was incubated at 0° to 2°C with hyaluronidase (*8, 9*). It was then centrifuged at 100,000 *g* for 85 minutes. The pellet and the lipid layer (pellicle) were discarded, and the remaining clear orange solution was designated whole EE. The EE was either fractionated immediately, or it was kept overnight in the refrigerator and then fractionated (*10*) on a column (2.5 by 80 cm, 400 ml capacity) of Sephadex G-25 (*11*).

Fractionation was carried out at 4° to 6°C at flow rates of 6 to 10 ml/min. Approximately 80 ml of EE was applied to the column and eluted with Hanks' solution (without phenol red). The excluded, high-molecular-weight fraction (H) was that part of the eluate containing hemoglobin as determined visually or by spectrophotometric readings at 410 and 540 mμ. The retarded or low-molecular-weight fraction (L) included the remainder of the fractions up to and including that with the first visible trace of phenol red. The two fractions were sterilized by being forced under pressure through HA or GS Millipore filters, or they were incorporated directly into media which were sterilized as described above after overnight storage at 0° to 2°C. Fractions have been stored up to 2 months in a liquid-nitrogen refrigerator without change in activity. Percentages of L and H were corrected for dilution during fractionation; they refer to the volume of original sample; for example, 5 percent H represents the same concentration of high-molecular-weight components which would be present in a medium containing 5 percent whole EE by volume. Cells were cultured in Ham's medium F10 (*12*) except that twice the amino acid concentrations were used, and supplements of 0.5 to 1.0 percent bovine serum albumin (*13*) and 5 percent fetal calf serum were added.

Plating efficiency, clonal growth rate, and expression of differentiated function were the criteria for the evaluation of media incorporating Sephadex fractions of EE. Cell counts were made with hemocytometers. The criteria for cellular differentiation in chick pigmented retina cells were the degree

of pigmentation and the morphology of the pigment granules (*14*). In chick cartilage, the criteria were the production of an extracellular, metachromatic matrix and the demonstration of $(^{35}SO_4)^{2-}$ incorporation into the matrix (*15, 16*).

Culture media containing H or L fractions of EE promoted growth of cartilage and pigmented retina cells in both clonal and high-density monolayer cultures. Clonal growth curves of both cell types demonstrate that generation times of 16 to 24 hours were obtained with 2 to 7 percent H; generation times in 2 to 7 percent L fell in the range of 28 to 36 hours. Maximum growth rates were obtained in 2 percent H and 2 percent L for pigmented retina cells, and in 7 percent H and 10 percent L for cartilage cells.

L-containing media allowed expression of the differentiation of pigmented retina and cartilage cell clones. Clones of pigmented retina cells grown in 2 percent L formed round colonies with typical epithelial cell morphology and contained large amounts of black pigment (Fig. 1, a and b). Clonal and subclonal cultures of 5- to 7-day limb chondrocytes or 12- to 15-day sternal chondrocytes grown in 2 to 7 percent L produced an extracellular matrix which stained metachromatically with toluidine blue. The cells incorporated $(^{35}SO_4)^{2-}$ into insoluble matrix material and displayed a morphology (Fig. 2a) typical of cartilage cells (*17*). Differentiated cartilage cells in L often grew upward into the medium in the center of the colony and formed long projections surrounded by matrix.

In media supplemented with H at concentrations greater than about 1.5 percent, however, subclones of cartilage cells grew without detectable evidence of their differentiated properties during a 16-day culture period. The cells in 7 percent H were elongated and fibroblastic in appearance (Fig. 2b), made no metachromatic matrix, and did not incorporate autoradiographically demonstrable $(^{35}SO_4)^{2-}$. The components of H responsible for the suppression of detectable differentiation have been quantitated by counting the percentage of cartilage-making colonies obtained when differentiated cells were plated at clonal densities into media containing varying amounts of H (*15, 16*).

In media supplemented with more than 2 percent H, primary cultures of pigmented retinal cells and subclones from differentiated colonies assumed elongated colonial morphologies, rapidly lost their pigmentation, and be-

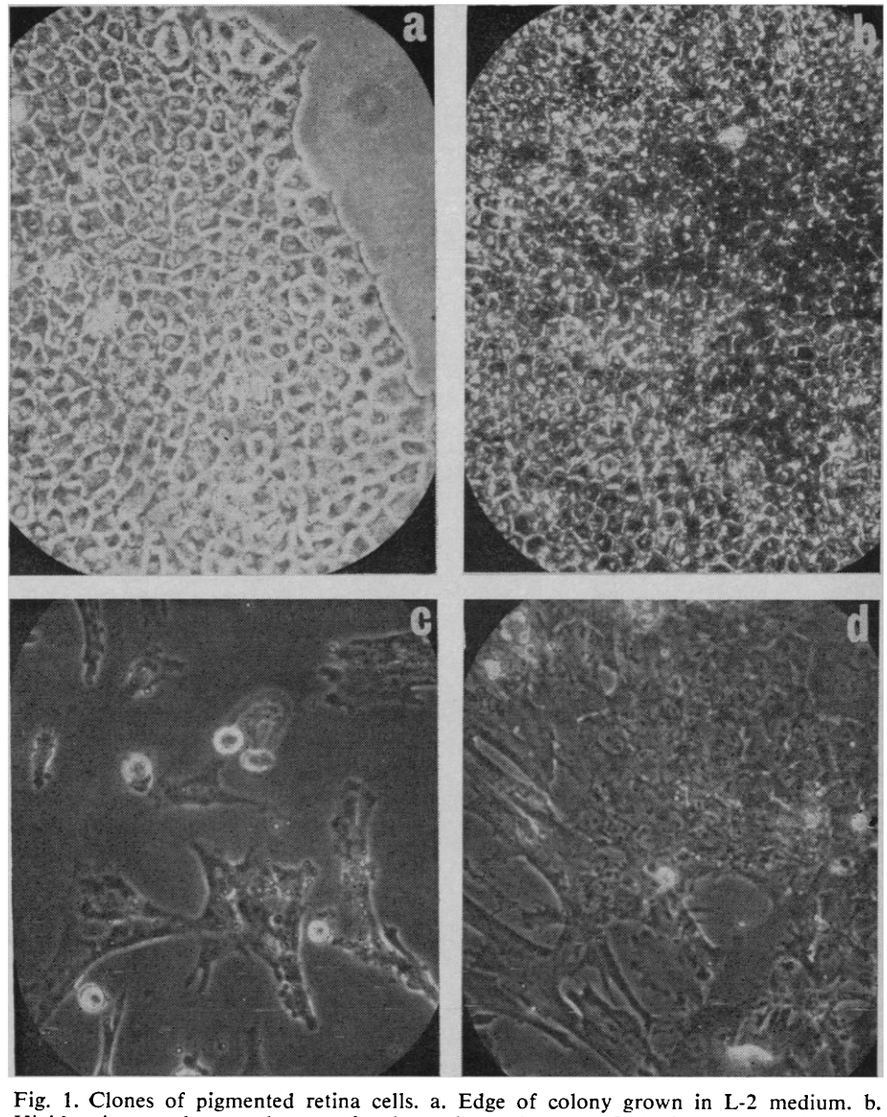

Fig. 1. Clones of pigmented retina cells. a. Edge of colony grown in L-2 medium. b. Highly pigmented, central area of colony shown in a. c. Cells from edge of colony grown in H-5. Note elongate "fibroblastic" morphology. d. Cells towards center of colony shown in c. Note prominent, ruffled membranes and highly spread nature of cells. Magnification, 250×.

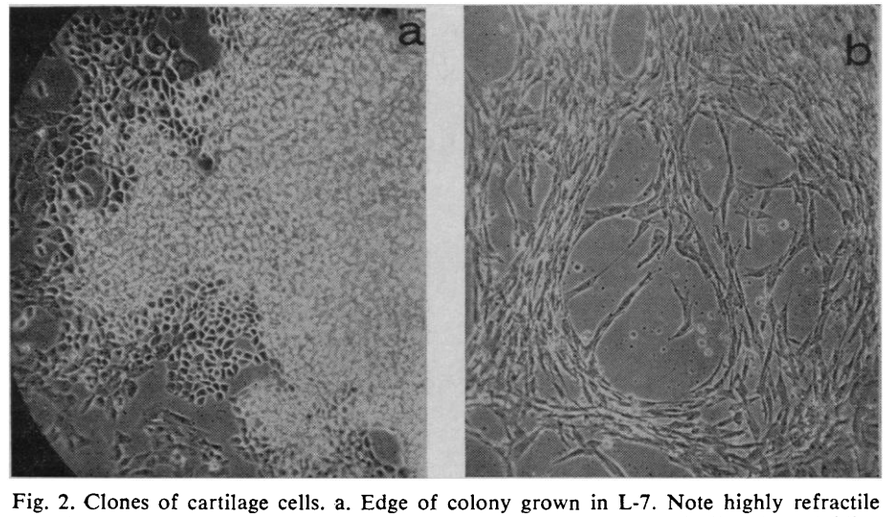

Fig. 2. Clones of cartilage cells. a. Edge of colony grown in L-7. Note highly refractile material towards center of colony. This material stains metachromatically with toluidine blue. b. Edge of colony grown in H-7. Note "fibroblastic" appearance of cells. Clones were derived from the same original parent clone. Magnification, approximately 150×.

came either fibroblastic in appearance or highly spread (Fig. 1, c and d). When replated in 2 to 7 percent L or in media containing low amounts of H (0.1 to 1.0 percent), both cell types displayed the same differentiated functions as the clonal cell cultures which were described in the preceeding paragraph [Fig. 1, a to d; Fig. 2, a and b (*14–16*)].

Expression of differentiated function in clonal platings of both cartilage and pigmented retina cells is equivalent in media without EE and in L-supplemented media. However, in media without EE, plating efficiencies and growth rates are usually lower than in either H- or L-supplemented media. Whole EE and H (as well as recombined H and L) inhibit formation of pigment and metachromatic matrix in proportion to concentration. Plating efficiencies for cartilage grown in media containing optimum amounts of L are equal to or higher than those of cartilage grown in all varieties of conditioned media tested.

Plating efficiences of cartilage cells grown in H were lower than those of cells grown in L-supplemented media (Table 1). Increasing the concentration of L from 2 to 12 percent increased growth rates but had little effect on plating efficiency. In 5 percent H, previously passed cartilage cell strains had only one-tenth the plating efficiency they had in 5 percent L (Table 2).

The cytotoxic (low plating efficiency) and inhibitory (to the expression of differentiation) properties of H can be reduced by conditioning the media, either by the procedure of Konigsberg (*6*) or by a shorter, 2- to 4-day conditioning period (Table 1). Higher plating efficiences and higher percentages of cartilage-making colonies were obtained in conditioned H media (or in conditioned, whole EE media) than in untreated H media.

Further fractionation of the substances responsible for the growth promoting, cytotoxic, and inhibitory properties of H on Sephadex have yielded the results summarized in Table 2. The H-fraction from G-25 was allowed to stand in the refrigerator (0° to 2°C) for 24 hours and then fractionated on G-25. The new H fraction thus obtained was applied sequentially to columns of successively higher grades of Sephadex (G-50, G-75, G-100). In each case two subfractions were obtained from the previous H fraction. The excluded H subfraction was reapplied to the next higher grade of Sephadex. The L fraction was tested without further treatment. A series of H subfractions, graded according to approximate molecular weights, was obtained. A portion of each subfraction was incorporated into medium (5 percent based on original whole EE concentration). The media were tested with clonal platings of a strain of cartilage cells derived from 50 pooled, cartilage-making colonies. Growth rates were estimated by counting the number of cells in 25 colonies on plates fixed 7 days after plating. Plating efficiencies and the percentages of cartilage-making colonies were determined from

Table 1. Effect of conditioned medium on the plating efficiency and differentiation of sternal cartilage cells from 13-day chick embryos. Plating efficiency and the percentage of differentiated colonies in conditioned media and fresh media were compared. Media were conditioned for 48 and 96 hours with 5×10^5 cells per 12 milliliters of medium in 100 mm plastic petri dishes. Muscle-conditioned medium was produced by the first and second passages of a culture of breast muscle from 11-day chick embryos; cartilage-conditioned medium was produced from parallel cultures of the tested strain at the fourth and sixth passage respectively. The test cloning plates were fed every third day by withdrawing half of the medium and replacing it with fresh L-7 medium. Plates were fixed on the 18th day of culture.

Medium		No. Colonies per plate		Cartilage-making colonies (%)	Plating efficiency (%)
Conditioning	EE Fraction % (v/v) in medium	Cartilage-making	Non-cartilage-making		
		48-hour-conditioning period			
Muscle	L-7	489	77		
		436	66	86.6	42.2
Cartilage	L-7	954	36		
		968	48	95.8	79
Cartilage	H-5	630	182		
		690	86	83.1	62.7
Fresh	H-5	184	195	48.5	30
Fresh	L-7	955	37	96.3	78.4
		96-hour-conditioning period			
Muscle	L-7	108	79		
		96	79	56.4	16.5
Cartilage	L-7	372	99		
		380	106	78.6	43.5
Fresh	L-7	424	82		
		454	73		
		465	92	84.5	48.2

Table 2. Assay of Sephadex fractions of embryo extract. The average generation time, plating efficiency, and percentage of differentiated colonies in clonal platings of sternal cartilage cells in media supplemented with 5 percent of the various fractions of EE were compared. HL denotes the components of H from the preceding column which were retarded on the specified column; HH denotes those components of H from the preceding column excluded on the specified column. The limits of the molecular weights for the subfractions are approximate; because proteins may be bound to the column by aromatic groups, the estimates of the molecular weights of the proteins are minimal. The G-100 HH subfraction was located by adding 0.5 mg of Blue Dextran 2000 (Pharmacia) per milliliter; phenol red was added to each new HH subfraction in order to locate the end of the succeeding HL subfraction. Plates were fed with the homologous test medium.

EE Fraction % (v/v) in medium		Molecular weight	Previously excluded on Sephadex Grade	Average generation time ± standard error (hr)	Plating efficiency (%)	Cartilage-making colonies (%)
Control (without EE)				78.0 ± 2.57	6.0	100
L-10	G-25	≦ 5,000		29.8 ± 0.89	36.3	100
L-5	G-25	≦ 5,000		35.8 ± 1.22	33.7	100
*HL-5	G-25	≦ 5,000	G-25	39.8 ± 1.27	24.4	100
HH-5	G-25	≧ 5,000	G-25	22.7 ± 1.11	2.2	0.9
HL-5	G-50	≧ 5,000 ≦ 10,000	G-25	28.1 ± 0.57	47.6	100
HH-5	G-50	≧ 10,000	G-25	21.5 ± 0.45	2.4	0.8
HL-5	G-75	≧ 10,000 ≦ 50,000	G-25 G-50	29.0 ± 0.40	39.3	99.9
HH-5	G-75	≧ 50,000	G-25 G-50	20.3 ± 0.44	5.25	22.6
HL-5	G-100	≧ 50,000 ≦ 100,000	G-25 G-50 G-75	21.2 ± 0.30	25.1	99.7
HH-5	G-100	≧ 100,000	G-25 G-50 G-75	19.3 ± 0.18	3.0	11.5
			Whole EE excluded on G-100 alone			
G-100	H-5	≧ 100,000		21.5 ± 0.42	3.3	10.4
G-100	H-5 (heated)	≧ 100,000		24.5 ± 0.32	53.6	99.8

* Refractionated after 24 hours.

plates fixed and stained on the 20th day of culture. All the subfractions of H promoted better growth than equal concentrations of L. Those subfractions which contained the components of highest molecular weight (G-100, excluded) were still effective inhibitors of expression of cartilage cell differentiation. Heat treatment (65°C for 10 minutes) of the G-100, excluded fraction before it was incorporated into the medium permitted the cartilage cells to express differentiated function. These cultures exhibited a slightly reduced growth rate, however (Table 2).

While the data do not rigorously exclude the possibility that H-supplemented media inhibit expression of the differentiated functions of cartilage and of retinal pigment cell by increasing the growth rate, cells with equally high average generation times (21.5 hours) may exhibit function or not, depending only on the absence or presence of the components of highest molecular weight (Table 2).

Growth-promoting factors are distributed among the high- and low-molecular-weight fractions of EE. Furthermore, a growth-promoting factor similar to that of the low-molecular-weight fraction (L) is spontaneously regenerated from the high-molecular-weight fraction (H, G-25) from which the low-molecular-weight components have previously been removed. If H (G-25) is allowed to stand for 24 hours in the refrigerator (2° to 4°C) and then refractionated on Sephadex G-25, a new L fraction as well as an H fraction are obtained (Table 2). The L fraction thus generated from H has growth stimulating properties similar to those of the original L fraction; the growth-promoting properties of the new H fraction are unchanged (*10*). Thus, part of the ability of H to promote growth may come from breakdown into or release of low-molecular-weight components from the large molecules in H. Similar suggestions have been made by Eagle and his collaborators (*18*) for the origin of the growth-promoting factors in serum. These workers have shown that the high-molecular-weight materials in serum, necessary for cellular growth (but not attachment), are broken down prior to utilization by the cells. Ultrafiltrates of digested serum can substitute completely for serum in most instances. Bound molecules may also be liberated from serum proteins and metabolized.

The inhibitory properties of EE may partly account for the dedifferentiation of primary cell cultures obtained in vitro by earlier workers (*5*, *17*). Similar inhibition of expression of differentiation is seen when cells are grown without EE but at high cell densities, at which they may get insufficient amounts of proper nutrients (*14*, *16*). The cells in these instances may generate inhibitory substances similar to those in EE. Conditioning of media containing whole EE or high concentration of H (*6*) definitely increases both plating efficiency and the percentage of differentiated clones; it does not improve L-supplemented media (Table 1). Conditioning may serve to add low-molecular-weight compounds from the large molecules in the medium. Conditioning may also inactivate or partially inactivate the high-molecular-weight, heat-labile, inhibitory factors in EE. For pigmented retina cells and cartilage cells the use of L has supplanted the use of conditioned media.

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Polysomes Extracted from Escherichia coli by Freeze-Thaw-Lysozyme Lysis

Abstract. *Polysomes can be extracted from* Escherichia coli *by freezing and thawing in the presence of lysozyme, followed by treatment with sodium deoxycholate. The method is simple and convenient; the yields consistently high.*

Several methods have been used to extract intact polysomes from bacteria: pressure cells (*1*), lysis of spheroplasts (*2*), and EDTA-lysozyme (ethylenediaminetetraacetate) treatment (*3*). These procedures have disadvantages: some are tedious or elaborate, and some give low or inconstant yields in terms of either RNA extracted or the proportion of the recovered ribosomes that are present in polysomes. We now describe a new method of breaking cells to provide high yields of polysomes quickly, consistently, and with greater convenience; it depends on the fact that freezing and thawing render *Escherichia coli* cells susceptible to the action of lysozyme.

In a typical experiment 30 to 100 ml of a log-phase culture of *E. coli* W, in tryptic digest medium (5×10^8 cell/ml), was poured over an equal amount of ice and centrifuged immediately for 5 minutes at 10,000*g*. The cells were resuspended in a plastic centrifuge tube in 0.5 ml of cold buffer (0.01*M* tris hydrochloride, *p*H 7.75, and 0.015*M* magnesium acetate) containing 0.5 mg of lysozyme. The suspension was frozen in acetone and solid carbon dioxide and then thawed carefully in cool water until the last bit of ice melted. After two such cycles, lysis was completed by incubation with 0.015 ml of 10-percent sodium deoxycholate for 3 minutes at 0°C. (Further cycles of freezing and thawing did not perceptibly improve the yield of RNA.)

The released DNA and cell debris were sedimented by contrifugation for 10 minutes at 30,000g, and the supernatant was analyzed by zonal centrifu-