*CONTACT INHIBITION, MACROMOLECULAR SYNTHESIS, AND POLYRIBOSOMES IN CULTURED HUMAN DIPLOID FIBROBLASTS**

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The contact inhibition of locomotion in human cells has been described by Abercrombie.[1] When normal diploid fibroblasts growing on a glass surface come into contact, an adhesion forms and cell movement in that direction stops. As the resulting "monolayer" of diploid cells becomes confluent, their growth rate also decreases markedly.[1, 2] In consequence, an upper limit is set for the population density of a given diploid cell culture which is not determined by properties of the medium. Human cell lines in which contact inhibition of growth is not operative (in general, heteroploid cells) form multilayered sheets in stationary culture, and attain population densities up to ten times those of human diploid cells.

The present communication describes some of the molecular events associated with the decreasing growth rate of cultured human diploid cells as they become confluent. The possible causal role of cellular contact is also considered.

Methods.—Cell strains: Four human diploid cell strains were studied: *embryonic bone,* isolated by Dr. Thomas G. Ward from a 16-week male embryo; *Bal 3,* cultured in this laboratory from the skin of a 4-year-old hyperglycinemic male;[3] *F1,* similarly cultured from the skin of a normal six-month-old male; and *Detroit 510,* cultured by C. S. Stulberg from the skin of a 9-month-old galactosemic female. All four strains were fibroblastic in appearance, euploid, and resembled the human diploid cell strains described by Hayflick.[4] All strains were checked periodically for the presence of PPLO and found to be uncontaminated.

Maintenance of cultures: Diploid cell cultures were maintained on a minimal medium,[5] supplemented with 5% calf serum and 7% fetal calf serum. Cultures were refed twice weekly, and on the day before subdivision or experimental use. More frequent feeding had no effect on the rate of cellular growth or the development of contact inhibition. Confluent monolayers were subdivided by treatment with trypsin (2 × crystallized, Worthington Biochemicals, 0.2% in 0.1 *M* Tris buffer, pH 7.7) for 1–2 min, rinsing with lima bean trypsin inhibitor (Worthington Biochemicals, 0.02–0.08% in 0.85%, NaCl), and finally dislodging the cells by shaking into the growth medium described above, followed by gentle pipetting. For serial propagation, confluent cultures were divided twofold. Counted inocula were used in the actual experiments.

Incorporation experiments: Replicate inverted T30 flasks[6] were inoculated with a total of 300,000 cells in 6 ml of growth medium. Fully grown cultures were used for this inoculum. At appropriate time intervals, flasks were used in duplicate for hemocytometer cell counts, total protein determination,[7] and for pulsing with C^{14}-labeled algal hydrolysate, uridine-2-C^{14}, and thymidine-2-C^{14} (New England Nuclear: specific activities 1430, 124, and 150 μc/mg, respectively). (See Fig. 1.)

Preliminary to pulsing with radioactive algal hydrolysate, the monolayer cultures were incubated for 45 min in serum-free growth medium, with the amino acids at 1/20 their usual concentration in order to reduce the size of the intracellular pool.[5, 5a] The cells were pulsed for 10 min in 6 ml of a similarly diluted medium containing 5 μc of C^{14}-algal hydrolysate. Incorporation was stopped by decanting the pulsing medium, washing the monolayer twice with cold Earle's salt solution, and dissolving the cells in 1 ml of 1 *N* sodium hydroxide. After 10 min at room temperature, 1 ml of 40% TCA (w/w) containing 2% casamino acids (Difco) was added to precipitate cell protein. The precipitate was collected on Millipore filters and counted in an end-window gas flow counter (background 2 cpm, efficiency 35%) to a standard error of less than 5%.

To measure the rate of RNA synthesis, cultures were overlaid for 30 min at 37° with 6 ml of

medium containing 1 μc uridine-2-C^{14}, washed as above, and dissolved in 1 ml of 2% sodium dodecyl sulfate. For the rate of DNA synthesis, cells were similarly incubated with 1 μc thymidine-2-C^{14} for 90 min, washed, and dissolved in 1 ml of 0.25 *N* sodium hydroxide. In both cases, TCA (1 ml of a 25% solution) was added to precipitate nucleic acids, and the samples were kept on ice until plated for counting as described above.

Detection of free cytoplasmic polyribosomes: Growing (1-day) and fully grown (8-day) cultures were examined for their free polyribosome content by sucrose density gradient centrifugation (Figs. 2 and 3, Table 1). Monolayers were pulsed with C^{14}-algal hydrolysate as described above, chilled, and harvested by trypsinization in the cold. The cells were resuspended in hypotonic buffer,[8] homogenized, and the nuclei separated from the cytoplasm by centrifugation.[9] The cytoplasmic fraction was layered onto 15–30% sucrose density gradients,[10] which were centrifuged, fractionated, and analyzed for absorbancy and isotope incorporation as previously described.[8]

Analysis of cellular RNA: Growing (1-day) and fully grown (8-day) cultures were pulsed with uridine-2-C^{14} and harvested as above (Table 2). Nuclear and cytoplasmic fractions were prepared, treated with sodium dodecyl sulfate, and subjected to sucrose density gradient centrifugation.[10] In all experiments, isotope incorporation data have been expressed as counts per unit cell protein or number in order to compensate for differences in cell populations.

Rate of Incorporation of C^{14}-labeled Amino Acids, Uridine, and Thymidine as a Function of Culture Age.—The incorporation of C^{14}-labeled amino acids, uridine-2-C^{14}, and thymidine-2-C^{14} in various stages of culture growth is illustrated in Figure 1 for the Detroit 510 fibroblast. Similar results have been obtained with the Bal 3 fibroblast. It is apparent that the rates of precursor incorporation into DNA, RNA, and protein decreased progressively as the cultures grew. On the eighth day, by which time a confluent monolayer had formed, the rate of cellular growth, measured by either cell count or cell protein, was 9 per cent of that on day 1 (24 hr after inoculation); and the incorporation rates per cell for uridine-2-C^{14}, thymidine-2-C^{14}, and C^{14}-algal hydrolysate were 14, 7, and 27 per cent, respectively, of those on day 1. The precipitous fall in the rate of uridine-2-C^{14} incorporation on day 3 was followed by a marked decrease in growth rate. The rate of thymidine-2-C^{14} incorporation, however, began to decrease on the second day after inoculation, while the cells were still growing actively.

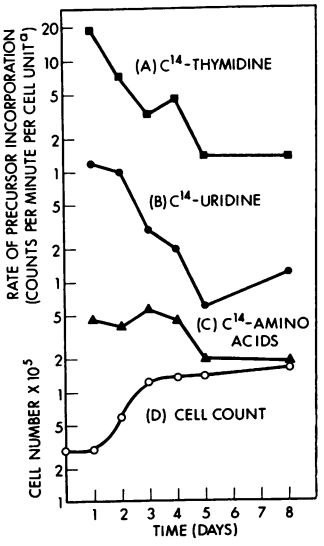

FIG. 1.—Rate of incorporation of C^{14}-labeled amino acids, uridine, and thymidine as a function of culture age. Cultures were pulsed at appropriate time intervals with thymidine-C^{14} (*A*, ■——■), uridine-2-C^{14} (*B*, ●——●), and C^{14}-algal hydrolysate (*C*, ▲——▲). Growth was measured by cell counts (*D*, O——O) and total protein determinations (*Methods*).[a] (Rates of incorporation are expressed as TCA-precipitable cpm per 10^3 cells (curve *A*) or per 10^2 cells (curves *B* and *C*).)

A complicating factor in interpreting the curves of Figure 1 may be the initial trauma of trypsinization and the attendant loss of cell substance.[11] The high rates of precursor incorporation in the first 24–48 hr may therefore be due in part to the repair of damaged structures, and the following decrease in incorporation not altogether attributable to the saturation of the developing culture. Also, the development of asynchrony after an initial period of synchronous growth may account for part of the drastic decrease in the rates of DNA and RNA synthesis. This

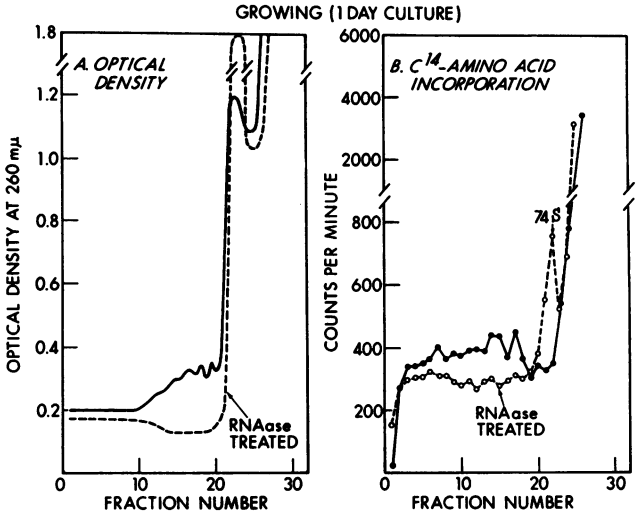

FIG. 2.—Free cytoplasmic polyribosomes in growing (1-day) cultures of embryonic bone. A growing (1-day) culture of embryonic bone was pulsed for 10 min with C^{14}-algal hydrolysate and analyzed by density gradient centrifugation to detect free polyribosomes (*Methods*, p. 351). Before centrifugation, half the cytoplasmic extract was treated with 10 γ ribonuclease (Worthington) (- - - in *A*, and O- - -O in *B*). The other half of the extract was untreated (—— in *A*, and ●——● in *B*).

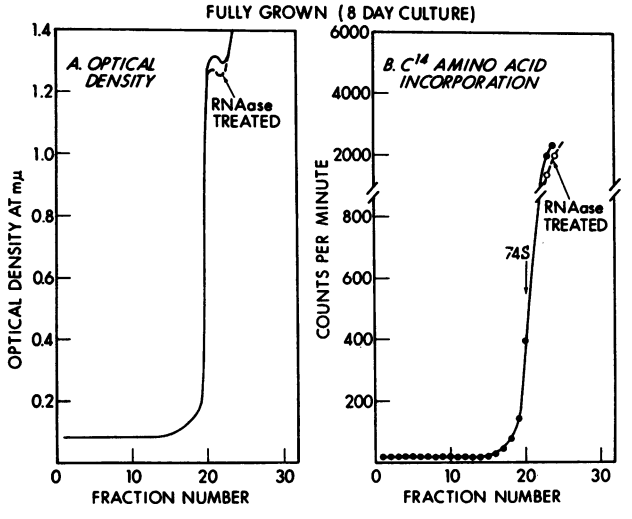

FIG. 3.—Free cytoplasmic polyribosomes in fully grown (8-day) cultures of embryonic bone. A fully grown (8-day) culture of embryonic bone was pulsed for 10 min with C^{14}-algal hydrolysate and analyzed by density gradient centrifugation to detect free polyribosomes (*Methods*, p. 351) Before centrifugation, half the cytoplasmic extract was treated with 10 γ ribonuclease (Worthington)(- - - in *A*, and O- - -O in *B*). The other half of the extract was untreated (—— in *A*, and ●——● in *B*).

may be resolved by autoradiography experiments now in progress. An apparent anomaly in the curve for amino acid incorporation is also of interest. Although the rate of cellular multiplication and of *net* protein synthesis decreased by a factor of 10 in the course of 8 days' growth, the amino acid incorporation decreased

by a factor of only 3.7. Most of the residual amino acid incorporation in the 8-day culture, then, must stem from processes other than *net* protein synthesis, and presumably from protein turnover.[12, 13]

Free Cytoplasmic Polyribosomes in Growing and "Inhibited" Cultures.—The cellular structure on which protein is synthesized has been shown to be the polyribosome, an aggregate of ribosomes held together by messenger RNA;[8, 10, 14–18] and when cells were growing actively 24 hr after inoculation, an appreciable number of polyribosomes were in fact found free in the cytoplasm of the diploid cells (Fig. 2). In the 8-day fully grown culture, however, more than 90 per cent of those free cytoplasmic polyribosomes had disappeared (Fig. 3). Figures 2 and 3 illustrate results in embryonic bone cells; qualitatively similar results were obtained with the Detroit 510 and F1 cultures.

When growing Detroit 510 cells were pulsed with C^{14}-algal hydrolysate for only 1 min (instead of 10 min as in Figs. 2 and 3), amino acid counts were again present in

TABLE 1

C^{14}-AMINO ACID LABELING OF GROWING AND FULLY GROWN CULTURES

Gradient region → Approximate S value →		"Pellet" >255S	"Polyribosomes" 255–95S	"Single ribosomes" 74S
		Total cpm in growing culture*		
Effect of ribonuclease on TCA-precipitable radioactivity	Control	645	320	56
	Treated	520	86	550
	Decrease	125	234	—
	Increase	—	—	494
		Total cpm in saturated culture*		
Effect of ribonuclease on TCA-precipitable radioactivity	Control	444	48	47
	Treated	319	32	129
	Decrease	115	6	—
	Increase	—	—	82

* Normalized on basis of protein content relative to that of saturated culture.

Growing (1-day) and fully grown (8-day) cultures of Detroit 510 cells were pulsed for 1 min with C^{14}-algal hydrolysate and analyzed by density gradient centrifugation to detect free polyribosomes as described in *Methods*. One half of the cytoplasmic extract was treated with ribonuclease as indicated in Fig. 2, and the other half was untreated. Sums of the radioactivity under the optical density peak for single ribosomes, in the polyribosomal region of the gradient, and in material with S values greater than 255 are given for both ribonuclease-treated and untreated gradients. The result of ribonuclease treatment is a decrease in the radioactivity in the "pellet" and "polyribosomes," and an increase in the "single ribosomes."

the polyribosome area (Table 1), but with no significant number of counts in the single ribosome peak; and on treatment of the cell homogenate with ribonuclease most of the counts in the polyribosome area (along with material absorbing at 260 mμ) were transferred to the single ribosome peak, with little residual background radioactivity or 260 mμ absorbancy. When fully grown cultures were similarly pulsed for 1 min, there was no significant optical density peak in the polyribosome region, and no significant radioactivity in either the poly- or single-ribosome area. Treatment with ribonuclease before centrifugation in this case did not change the optical density profile or the distribution of radioactivity in the polyribosome area. The slight increase in the single ribosome peak probably represents material transferred from the "pellet."

Free cytoplasmic polyribosomes also disappear after prolonged starvation[19] or actinomycin treatment[10] of cultured mammalian cells, and during sporulation of Neurospora.[20] A similar disappearance of free polyribosomes in saturated multilayered cultures has been noted in electron micrographs of heteroploid mouse

fibroblasts.[21] In HeLa cells, material staining with toluidine blue and presumed to be polyribosomes also disappears from the cytoplasm of fully grown cultures.[22]

The continuing significant incorporation of amino acids into fully grown cultures of diploid fibroblasts in the absence of demonstrable free cytoplasmic polyribosomes is under continuing study. Preliminary experiments suggest the presence of an amino acid-incorporating system in larger cellular structures, which may be related to protein turnover.

RNA Synthesis in Growing and "Inhibited" Cultures.—Several possible causes may contribute to the absence of free polyribosomes in the older cultures: breakdown or deficient synthesis of ribosomes or of the linking messenger RNA; the failure of single ribosomes and messenger RNA to aggregate into polyribosomes; or the accelerated breakdown of the formed polyribosome. In relation to the first possibility, pulse experiments with uridine-2-C^{14} have shown that the decreased rate of RNA biosynthesis in fully grown cultures involves most of the RNA species (Table 2). The minor differences there shown, and the progressive development of this

TABLE 2

RNA SYNTHESIS IN GROWING AND FULLY GROWN CULTURES

	% Precipitable Counts in Indicated RNA Fraction							
	Nucleus				Cytoplasm			
	30′ pulse		90′ pulse		30′ pulse		90′ pulse	
RNA species	1-day culture	8-day culture	1-day culture	8-day culture	1-day culture	8-day culture	1-day culture	8-day culture
4S	21	22	18	16	84	76	45	58
16S	14	25	18	33	5	9	33	31
28S	23	32	33	27	3	5	17	9
35–45S	34	21	30	25	—	—	—	—

Growing (1-day) and fully grown (8-day) cultures of embryonic bone were pulsed for the indicated time periods with uridine-2-C^{14}. Nuclear and cytoplasmic fractions were prepared and analyzed by density gradient centrifugation (see *Methods*). In these experiments, the *absolute* rate of incorporation of uridine-2-C^{14} in the growing (1-day) and saturated (8-day) cultures differed by a factor of 16 per unit cell number (cf. p. 351 and Fig. 1).

inhibition as growing cells come into contact are under study, in the hope of observing early differential effects on the several RNA species.

Discussion.—The results here reported show that, despite continuing active cellular metabolism,[4, 23] macromolecular synthesis is progressively depressed as monolayer cultures of human diploid cells become confluent. This inhibition is completely reversible, in that the rate of precursor incorporation increases as soon as 7 hr after subculture,[19] and as much as 15-fold in the first 24 hr. This progressive inhibition of macromolecular synthesis, and its reversal on subdivision, are associated with the disappearance and reappearance of free cytoplasmic polyribosomes.

Abercrombie[1] has shown that cellular contact inhibits the motility of diploid fibroblasts in culture; and Goldé[2] has suggested that mitosis similarly is subject to contact inhibition. A reasonable working hypothesis is that these phenomena are interrelated: that cellular contact initiates a series of intracellular reactions, ultimately expressed in the inhibition of macromolecular synthesis, cessation of motion, and inhibition of mitosis.

The possibility of controlling humoral factors released into the medium appears to be excluded by the findings of several laboratories. Abercrombie[1] found that in a single culture vessel new cells growing out from two separate foci of contacted cells exhibit contact inhibition when they meet. Todaro *et al.*[24] found that the mitotic

index of growing cells is unaffected by the presence of contacted nongrowing cells in the same culture vessel. Similarly, when fully grown and freshly inoculated cultures were paired[12] and overlaid with the same medium, the latter grew at a normal rate until they also formed a complete monolayer.[19]

Summary.—In cultures of human diploid fibroblasts, the rates of DNA, RNA, and protein synthesis per cell are progressively depressed as the culture becomes confluent, reaching levels 5–15 per cent of those in the freshly inoculated culture in the case of DNA and RNA, and 30–50 per cent in the case of protein. These decreases are associated with the disappearance of most of the free cytoplasmic polyribosomes. The changes are completely and rapidly reversible on subdivision of the culture.

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The following abbreviations have been used: TCA = trichloroacetic acid; RNA = ribonucleic acid; DNA = deoxyribonucleic acid.

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[13] This hypothesis is supported by some quantitative considerations. The 24-hr generation time in the initial (growth) phase of this experiment implies a rate of *net* protein synthesis of approximately 3%/hr; and a superimposed protein turnover rate of 0.7–1%/hr[12] would give a total rate of amino acid incorporation of approximately 4%/hr. Since *net* protein synthesis in the essentially nongrowing 8-day culture cell is reduced by a factor of 10, to 0.3%/hr, while turnover remains at 1%/hr,[12] the observed rate of amino acid incorporation in the fully inhibited culture should be approximately 1.3%/hr, 1/3 that in the growing cell. The observed ratio was 1/3.7.

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*MOLECULAR SIZE AND CIRCULARITY OF DNA IN CELLS OF MAMMALS AND HIGHER PLANTS**

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The idea that DNA might be organized as series of rings in chromosomes of higher organisms was first elaborated by Stahl on genetic grounds.[1] The credibility of this viewpoint is enhanced by physical demonstrations of circular DNA in some bacteria and viruses,[2, 9] and by the self-regulation of selective strand transcription in DNA circles as revealed by the studies of Spiegelman and co-workers.[3] This report presents two pieces of evidence relevant to theories of chromosome organization: (1) that circles can be found in DNA from mammalian cells, and (2) that the native molecules of DNA in higher plants and animals vary markedly in size.

The studies here reported were carried out on isolated wheat nuclei and boar sperm. The first material was chosen because nuclei could be readily isolated from dormant wheat embryos in nonaqueous media[4] since such embryos are naturally dehydrated. The second material was chosen after experience with wheat nuclei indicated that the abundance of nonhistone protein in such nuclei required considerable mechanical manipulation in order to effect extraction of the DNA.

Methods.—Isolated wheat embryos were disintegrated at temperatures not exceeding 25°C by blending without the addition of solvent in an "Omnimixer" (Servall). In the course of blending, the dry powder was periodically sifted through a 35-μ nylon mesh, the retained portion being returned to the Omnimixer. Nuclei were then isolated by use of cyclohexane-carbon tetrachloride mixtures. Such preparations were stored in 95% ethanol after a series of washes with ethyl ether, ethanol:ether (1:1), ether, ethanol:ether (2:1), and ethanol. A stock solution of the protease, Pronase (Calbiochem), was prepared at a concentration of 2 mg/ml. The pH was adjusted to 5 with HCl, heated to 80°C for 10 min, cooled, the pH readjusted to 7.0 with NaOH, and solid sodium chloride added to a concentration of 1.0 *M*. The solution thus prepared was DNase-free and could be stored for at least several months at −20°C without loss in activity.

Defatted wheat nuclei were suspended in a solution adjusted to pH 8 and containing the following components: 1% cetyltrimethylammonium bromide ("cetavlon"), 1.0 *M* NaCl, 0.01 *M* ethylenediaminetetracetic acid ("versene"), and 0.01 *M* Tris buffer. This solution, less the cetavlon, will henceforth be referred to as "buffered saline." The suspension of nuclei was maintained at 0°C for 20 min, diluted with an equal volume of 0.01 *M* versene-Tris (pH, 8), the fibers were collected with a glass rod and transferred to buffered saline solution. After gentle overnight agitation at 0°C, an equal volume of stock Pronase was added and the mixture incubated at 60°C for 4 hr. The suspension was cooled to 37°C, crystalline RNase added to a concentration of 0.4 mg/ml, and incubated for 30 min.[5] The Pronase treatment was then repeated. All digestions were carried out in a dialysis bag suspended in buffered saline solution. After the final incubation the suspension was clarified by centrifugation, the supernatant fluid containing the DNA. To remove cetavlon from the DNA, one alcohol precipitation step was introduced into the procedure