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pH as a Determinant of Cellular Growth and Contact Inhibition

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ABSTRACT **1) Both the growth rate and the maximum population density of several normal, virus-transformed, and cancer cells were markedly pH-dependent; the optimum varied from pH 6.9 to 7.8. At the optimum pH, some diploid human cells attained population densities comparable to those of cancer or virus-transformed cells. Contact inhibition of growth is facilitated by repeated fluctuations of pH in nonphysiological ranges, and may not be an intrinsic and necessary attribute of diploid cells in culture. 2) At pH 8.3, at which there was little or no cellular multiplication, the protein content per cell increased 2- to 5-fold over a period of 10-16 days, and was slowly reversed to normal concentrations on restoration of pH to the optimal range. 3) Uridine uptake by contact-inhibited human cell cultures was stimulated by refeeding with salt solution, and to the same extent as by complete (serum-supplemented) growth medium; that immediate increase did not involve the reinitiation of cellular growth and multiplication. Contact inhibition was, however, reversed in 2-4 days by an appropriate increase in the serum concentration of the medium.**

Normal cells in culture, unlike cancer cells or virus transformants, show "contact inhibition" of growth, i.e., the population density stabilizes at relatively low levels (1, 2), the precise value varying with the individual cell and the serum (2, 3). This population-dependent inhibition of growth is associated with a marked decrease in the rate of RNA, DNA, and protein synthesis, and a diminution in the number of free cytoplasmic polyribosomes (4). The changes are rapidly reversed when the culture is subdivided.

Simple refeeding of contact-inhibited 3T3 cultures from mice has been reported to cause an increased incorporation of labeled uridine into Cl_3CCOOH-insoluble material within 30 min, due to either increased synthesis of RNA (5) or increased transport of the exogenous labeled uridine (6). As will be shown here, however, an initial increase in both uridine transport and incorporation can be produced in contact-inhibited cultures by refeeding with serum-free medium, or even with salt solution. In at least one cell line, simple alkalinization of the old medium sufficed to stimulate uridine uptake. This observation has led to the finding that the growth rate of diploid, virus-transformed, or cancer cells is markedly pH-dependent, as is also the maximum population density attained. "Contact inhibition" of growth may be determined not only by population density, but by artefactual variations in the pH of the medium.

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MATERIALS AND METHODS

Cell strains

Of the four human diploid strains studied, two were skin fibroblasts, Penny D (7) and MS2A (8), and two were lung fibroblasts, WI 38 (9) and KL2.† Five aneuploid cells were also studied, an SV40-transformed human cell [WI 26 VA (2)], a human cancer cell (HeLa-AT; American Type Culture Collection), an adeno-transformed monkey kidney cell [GMK-adeno (2)], a mouse fibroblast [3T3 (10)], and a rabbit lens cell (11). All were maintained in antibiotic-free media to assure against mycoplasma contamination (12), and were monitored periodically for the absence of mycoplasma.

Maintenance of cultures

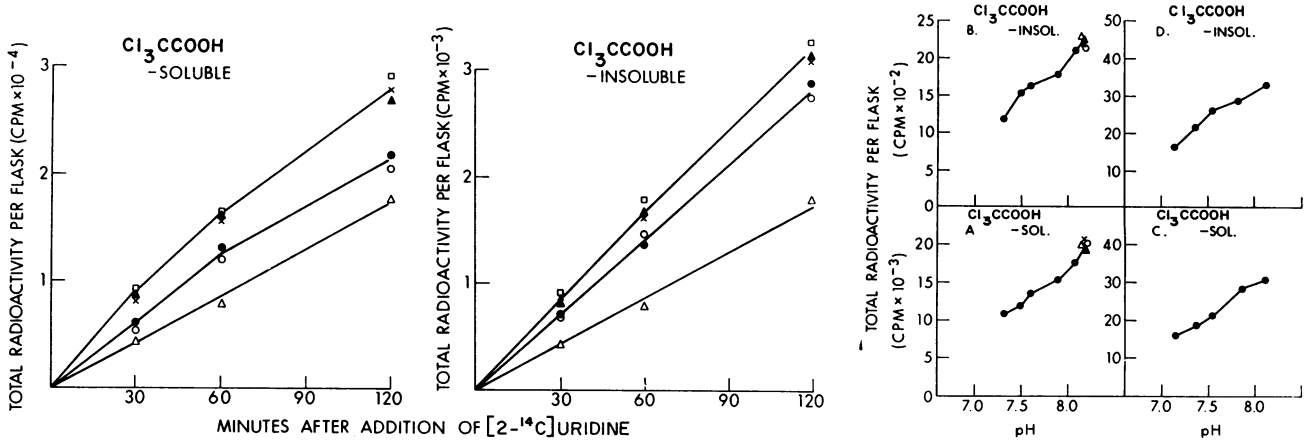
All cultures were grown in a minimum essential medium (13), supplemented with 5% calf serum and 5% fetal-calf serum. Stock cultures were fed every other day, and on the day before subdivision or an experiment. The cells were subdivided and maintained as described (1, 2).

[2-^{14}C]Uridine incorporation

Replicate T-15 flasks (14) were inoculated with 150,000–180,000 cells in 3 ml of growth medium. Cultures were refed after 2 days, and daily thereafter. At appropriate time intervals, cell protein (15) and cell number (with a Coulter counter) were determined, each in duplicate. When the cell number did not vary significantly in at least two consecutive counts, 48-hr apart, the cultures were allowed to stand without refeeding for 48–96 hr before the uridine-labeling experiment.

Cultures were overlaid at 37°C with 3 ml of medium containing 0.08–0.12 μCi [2-^{14}C] uridine (Schwarz BioResearch, Inc., diluted to a specific activity of 20 μCi/μmol for experimental use). At the end of the labeling period, the medium was withdrawn and saved for analysis. In most experiments, only 15–20% of the uridine in the medium had been taken up by the cells in a 2-hr incubation. The cells were washed twice with ice-cold Earle's (salt) solution, drained, and fixed with 3 ml of cold 8% Cl_3CCOOH. After about 30 min, the fluid was withdrawn, filtered, and neutralized, and 0.5 ml was counted in a Beckman scintillation counter in 10 ml of Bray's, or toluene–Triton X, solution. The cells were again washed 3 times with cold Earle's solution, drained overnight, and dissolved in 3 ml of 0.05 M Na_2CO_3–0.025 M NaOH, before protein determination and scintillation counting.

† Embryonic human-lung fibroblast, initiated in this laboratory by Dr. E. Levine.



(*Left*) FIG. 1. The stimulation of [2-^{14}C]uridine uptake and incorporation by refeeding "contact-inhibited' MS2A cells.

	Complete medium
Δ Control	Δ Serum-free
○ Serum only, 0.3 ml	□ 10% serum
● Serum only, 0.6 ml	× 20% serum

(*Right*) FIG. 2. Stimulation of [2-^{14}C]uridine uptake and incorporation in contact-inhibited MS2A cells by alkalinization of medium (*A* and *B* with NaOH; *C* and *D* with Hepes–Tricine buffers). All counts after 1 hr at 37°C. ○ = serum-free growth medium; Δ = serum-free medium, omitting amino acids; × = serum-free medium, omitting vitamins; ▲ = Earle's salt solution.

pH measurement

pH was measured in a Beckman *Zeromatic II*, at 25°C. The actual pH at 37°C was therefore slightly (<0.1 pH unit) higher than those indicated in the tables and figures.

Growth as a function of pH

To grow cells at a reasonably constant pH, the bicarbonate of the medium (26 mM) was supplemented with nongaseous buffers: 20–50 mM "Hepes" (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) and 5–10 mM "Tricine" (*N*-tris-hydroxymethyl methylglycine) (Calbiochem). The exact concentrations used were determined by their toxicity for the specific cell strain.

Within the pH range 6.7–8.4, this buffer provided a reasonably stable pH (±0.2 pH for 24 hr, except in heavy cultures). Replicate cultures were grown at 37°C in stoppered T-15 bottles, and were refed daily during the course of the experiment. Cell number, cell protein, and medium pH were determined at appropriate intervals.

To control the possibility that the observed effects might have been due to the specific buffer additives, rather than to changes in pH, cells (MS2A) were grown in 60-mm Falcon Petri dishes in a CO_2 incubator at fixed partial CO_2 pressure, but with varying concentrations of $NaHCO_3$ in the medium (16), permitting the pH to vary from 7.0 to 8.35. Qualitatively, the same effects were observed as with the Hepes–Tricine buffers.

RESULTS

Stimulation of uridine incorporation in "saturated" cultures by refeeding

As shown in Fig. 1, when contact-inhibited MS2A cultures were refed with either serum-free medium or medium supplemented with 10–20% serum, or if additional fresh serum was added to the old medium, there was a linear increase in [2-^{14}C]-uridine incorporation over a period of at least 2 hr, beginning immediately after refeeding. The slopes for incorporation into Cl_3CCOOH-soluble and Cl_3CCOOH-insoluble material were almost identical, suggesting that the observed stimulation of [2-^{14}C]uridine incorporation was not due to increased RNA synthesis, but, as indicated by Cunningham and Pardee (6), to an increased uptake of labeled precursor.

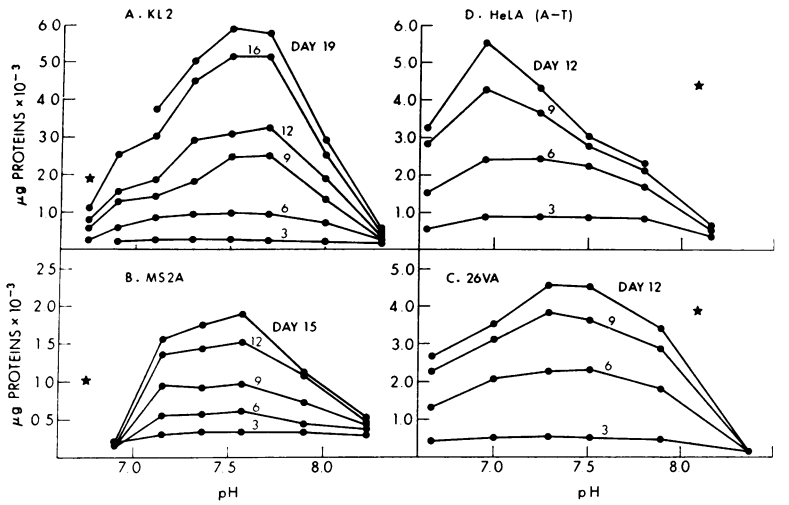
Unexpectedly, uridine uptake and incorporation were similarly stimulated if Earle's salt solution was used to refeed the contact-inhibited culture, rather than complete growth medium (see Fig. 2). Since refeeding with these bicarbonate-buffered media caused the culture to become more alkaline, the pH in saturated (contact-inhibited) cultures was adjusted with dilute alkali without changing the medium. The effect was qualitatively the same as that produced by the addition of fresh medium, with a direct correlation between pH and increased uptake into Cl_3CCOOH-soluble and Cl_3CCOOH-insoluble material (Fig. 2*A*, *B*). Similar data were obtained when the cultures were refed with fresh Hepes–Tricine-buffered medium, previously adjusted to pH 7.1–8.2 (Fig. 2*C*, *D*).

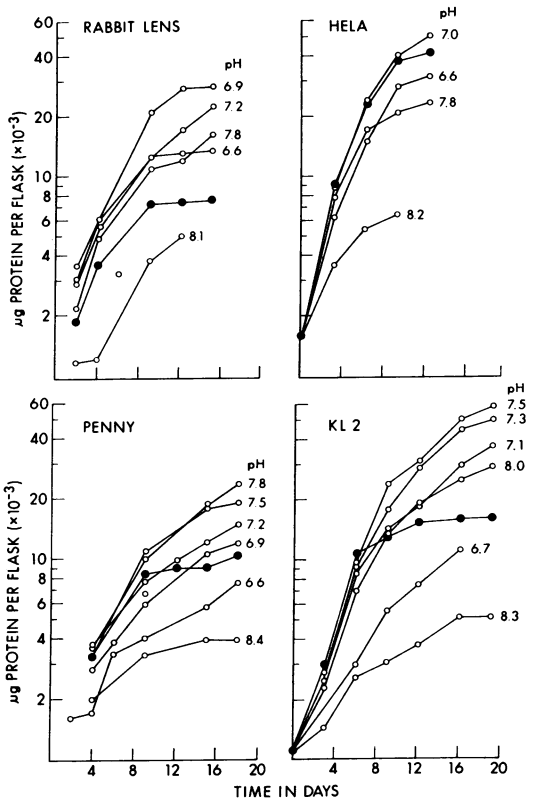
Uridine incorporation by strains Penny D and KL_2 was similarly stimulated by refeeding with fresh medium or salt solution, independent of the presence of serum. The increased incorporation could again be accounted for quantitatively by increased uptake of precursor. With these strains, however, alkalinization of the old medium did not result in increased uridine uptake.

In contrast to the results reported with mouse strain 3T3 (5), the initial increase in uridine uptake by contact-inhibited human cell cultures refed with fresh medium of the same composition was not followed by a measurable acceleration of either protein or DNA synthesis.

Effect of pH changes on rate of cellular growth and on the maximum population attained

The effect of pH on the growth (cell protein) of two human diploid strains (MS2A) and KL_2), a virus-transformed cell





(*Left*) FIG. 3. The growth of human cells as a function of the medium pH.

(*Right*) FIG. 4. "Contact inhibition" of growth in diploid human fibroblast and rabbit-lens cultures with fluctuating pH (●——●), and its absence at stabilized pH (○——○). (Note absence of contact inhibition with heteroploid HeLa cells).
●——●, Bicarbonate-buffered.
○——○, pH stabilized with organic buffers.

(WI 26 VA) and a cancer cell (HeLa A-T) are summarized in Fig. 3. With KL_2, the optimum growth, whether in terms of growth rate or maximum population density (Fig. 3*A*), was at pH 7.5–7.6. At that pH, the cultures attained population densities after 19 days of 65×10^4 cells and 390 μg of protein per cm^2, values 2.8 and 3.4 times greater than those observed with the same cell in ordinary $NaHCO_3$-buffered media (★ in Fig. 3), and of the same order of magnitude as those attained by HeLa cells and a virus transformant (see below). Qualitatively similar results were obtained with three other human fibroblast strains (MS2A, Penny D, and WI 38), an SV40-transformed human cell (WI 26VA), a human cancer cell (HeLa), an adenovirus-transformed monkey cell, a mouse fibroblast (3T3), and a rabbit-lens culture (Figs. 4, 5 and Table 1).

The pH optimum for growth varied from 6.9–7.0 for the WI 38, HeLa, and rabbit lens cells, to 7.8 for the Penny cell (Fig. 5, Table 1). At the optimum pH, the rabbit cells attained population densities 4 times greater than the maximum observed in bicarbonate-buffered cultures, in which the pH had fallen from 8.0 to 6.9 in the course of 24 hr (Fig. 4). There was usually little growth at pH 6.2, whether in terms of cell number or cell protein. At pH 8.3, on the other hand, although the cell number did not change significantly after the first 3 days, the cell protein per cell continued to increase slowly (Fig. 6), and by day 16, was as much as 5 times as great as in actively growing cells. This effect was reversed when the pH was restored to 7.5; i.e., cellular multiplication was reinitiated and

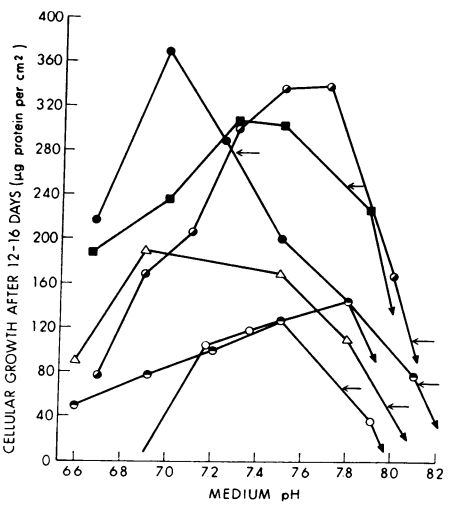


FIG. 5. pH optima for normal, cancer, and virus-transformed cells (the arrows in the figure indicate the growth attained in bicarbonate-buffered ("contact-inhibited") cultures, with widely fluctuating pH).

	Human	*Rabbit*
Normal	◐ KL ◓ (Penny) ○ (MS2)	Δ lens
Virus-transformed	■ WI 26VA	
Cancer	● HeLa	

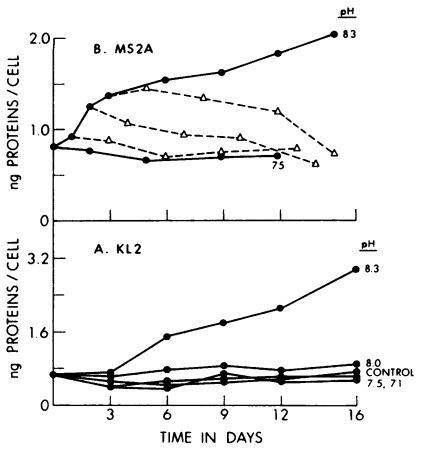


FIG. 6. Progressive increase in protein per cell at pH 8.3, and reversal (MS2A) to normal concentration at pH 7.5 (Δ- - -Δ) (control = bicarbonate-buffered medium with fluctuating pH).

the concentration of protein per cell returned to normal. However, the time required for this "recovery" was a function of the time for which the cells had been kept in alkaline medium: 5 days after a 24-hr exposure to pH 8.3, and 11 days after a 72-hr exposure (Fig 6*B*).

DISCUSSION

a. Stimulation of uridine uptake by refeeding contact-inhibited cultures

We have confirmed the finding of Cunningham and Pardee (6) that refeeding contact-inhibited cultures primarily stimulates the uptake of precursor, rather than the rate of RNA synthesis (5). We have, however, been unable to confirm the role of serum in that effect. With three different strains of human diploid cells, uridine uptake was stimulated whether the cells were refed with growth medium containing 20% serum, or with simple salt solution (Fig. 1); and with one cell strain, MS2A, increased uptake and incorporation could be induced merely by alkalinization of the medium, without refeeding (Fig. 2). These effects were, however, quantitatively trivial, and were not followed by a measurable increase in the synthesis of RNA, DNA, or protein. Further, in contrast to results reported with mouse-strain 3T3 (5), growth was not initiated in contact-inhibited human diploid cells by refeeding with medium that contained serum at the same concentration. However, contact inhibition was effectively reversed over a period of 48–72 hr by an increase in the serum concentration from (e.g.) 5 to 20% (unpublished data). The early stimulation of uridine uptake by refeeding with, for example, salt solution can thus be dissociated from the slow reversal of contact inhibition and the reinitiation of cellular growth by refeeding with higher concentrations of serum.

b. Effect of pH on growth

The rate of growth of all the cell lines studied, whether normal, cancer, or virus-transformed, as well as the maximum populations achieved, were markedly pH dependent. The optimum pH varied from 6.9 in the case of the rabbit lens cells, to 7.8 for human fibroblast, strain Penny. At those optima, cells attained densities 2–4 times greater than those attained under "normal" culture conditions (with fluctuating pH as a result of the use of bicarbonate buffers). At pH 8.3, there was unbalanced growth, in the sense of continuing protein synthesis, in the almost complete absence of cell division. In consequence, the protein content per cell increased to reach 2 to 5 times those concentrations observed under normal conditions. The slow reversal to normal values when the pH was restored to its optimum was preceded by increased DNA synthesis (unpublished results).

TABLE 1. *Optimum pH for the growth of several normal, cancer, and virus-transformed cells*

Cell type	Strain	pH for optimum growth*	Increased growth at optimum pH†
Human			
Normal	KL_2	7.5–7.7‡	3.2
	MS2A	7.6	1.9
	Penny	7.5–7.8‡	2.1
	WI 38	7.8	2.6
SV40-transformed	WI 26 VA	7.3–7.5‡	1.2
Cancer	HeLa	7.0	1.3
Rabbit lens		6.9	3.7
Mouse fibroblast	3T3	7.5–7.8‡	1.8
	L 929	7.0–7.5‡	1.0
Monkey			
Adenovirus-transformed	GMK-VA	6.6–7.2‡	0.9§

* Measured at 25°C (see Figs. 3 and 5).

† Relative to growth in bicarbonate-buffered medium, in which pH fluctuated from pH 8 to pH 7 after each refeeding. Data expressed as *times* growth in the bicarbonate medium.

‡ Essentially equal growth over the indicated pH range.

§ Ordinary bicarbonate-buffered medium was at the optimum pH during most of the culture cycle; combination of Hepes (20 mM) and Tricine (5 mM) was slightly toxic.

The practical significance of improving cellular yields by appropriate pH control requires no elaboration.

c. pH and contact inhibition

When human diploid cells are fed with bicarbonate-buffered growth medium supplemented with, e.g., 10% serum, there is an initial alkaline shift, with the highest pH (about 8.0) in about 30 min, followed by progressive acidification of the medium, over a period of 24 hr, to as low as pH 6.8; the rate and degree of these shifts vary with the specific cell and the population density. Such cultures eventually become "contact inhibited," i.e., the population density stabilizes with daily changes of medium. We have shown that if the pH of the medium is stabilized by the addition of nonvolatile organic buffers, this early contact inhibition is frequently not observed and the cells continue to grow for as long as 16–19 days,* when they begin to come off the glass surface (see Fig. 4). The rate of growth, and the maximum population density achieved are, however, markedly pH-dependent. The optimum pH varies with the cell strain, from 6.9 to 7.8. At that optimum, diploid human cells and a rabbit lens cell attained population densi-

* The generation time of KL2 at pH 7.5 averaged 1.5 days during the first 6 days of growth, and about 5.5–6.0 days from the 6th to 16th day. Corresponding values for strain MS2A were 2.6 and 5.7. Similarly progressive increases in cell doubling time were observed by Kruse and Miedema (17, 18) in perfusion cultures.

ties 2–4 times greater than those attained under "normal" culture conditions, in some cases approximating to the yields observed with virus-transformed and cancer cells (Figs. 4, 5).

The inhibition of macromolecular synthesis and cellular division in crowded cultures is therefore facilitated by a combination of cellular interaction and repeated fluctuations of pH in nonphysiological ranges. These results explain the finding of Kruse and Miedema (17, 18) that in perfused cultures, cells grow to population densities far exceeding those observed under ordinary culture conditions. In their experiments, also, pH varied only slightly; the population densities achieved were comparable to those reported here in appropriately buffered cultures with periodic refeeding. The artefactual aspects of the phenomenon of contact inhibition described here cast doubt on its relevance to the growth of cells *in vivo*, and suggest that the sustained multiplication of cancer cells *in vivo* may involve factors other than an escape from this type of growth control. It is, however, to be noted that even at stabilized and optimal pH, only a fraction of the diploid strains studied attained populations comparable to those achieved by malignant cells (Fig. 5). Studies are therefore in progress on the progressively retarded growth of normal cells at high population densities even at pH levels optimal for growth.

d. Possible other effects of pH

The striking effect of pH on the pattern of cellular growth and contact inhibition may extend to other properties of cultured cells. Thus, enzymatic activities or organ-specific functions, yields of cytolytic viruses, susceptibility to viral transformation, and efficiency of hybridization may be as susceptible to pH variation as is cellular growth itself.

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