pH AND POPULATION DENSITY IN THE REGULATION OF ANIMAL CELL MULTIPLICATION

H. RUBIN

From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

ABSTRACT

Sparse and dense cultures of chick embryo cells were affected differently by pH. The rates of cell multiplication and of thymidine-^{3}H incorporation into DNA of dense cultures were increased as the pH was increased from 6.6 to 7.6. At pH higher than 7.6 the rate of multiplication decreased slightly in the dense cultures, but the rate of thymidine-^{3}H incorporation continued to increase. The discrepancy was due in part to cell death and detachment at very high pH, and in part to a more rapid uptake of thymidine-^{3}H at very high pH. Sparse cultures were much less sensitive to pH reduction and, when a suitably conditioned medium was used to minimize cell damage, very sparse cultures grew almost as well at pH 6.7 as at higher pH. The rates of cell multiplication and thymidine-^{3}H incorporation at low pH decreased in the initially sparse cultures before they reached confluent cell densities. There was no microscope evidence of direct contact between plasma membranes of cells at these densities although the parallel orientation indicated that the cells were influencing locally each other's behavior. Even at much higher cell densities, electron microscopy revealed large intercellular gaps partly filled with a fragmentary electron-opaque material suspected to be glycoprotein. Wounding experiments showed that pH affected cell migration in a manner similar to its effects on cell multiplication. Low pH inhibited cell migration, but those cells which migrated into the denuded region multiplied as rapidly at low pH as at high pH. The effects of pH on growth were correlated with effects on the uptake of 2-deoxyglucose-^{3}H. Dense populations of cells inhibited by low pH were stimulated to incorporate thymidine-^{3}H by the addition of small amounts of diethylaminoethyl-dextran. Rous sarcoma cells at high cell density were less sensitive to pH than were normal cells at the same density, but were more sensitive than sparse normal cultures. The results suggest that cell growth is inhibited through the combined effects of both lowered pH and high cell density on cell surface permeability.

INTRODUCTION

The growth rate of chick embryo cells attached to a solid substratum decreases when the cells reach a high population density (12, 15–17). The population density at which growth retardation occurs is not precisely defined. It varies with type of cell, concentration of serum, and factors yet unknown. In the case of chick embryo cells in medium containing 1% serum at the physiological pH of 7.4, growth is not retarded until after the cell sheet has exceeded confluency; in higher serum concentrations, the medium is depleted before cell growth stops (14).

One variable which has not been much studied is the effect of pH on the growth of different concentrations of cells (3, 8). In a preliminary report (14) I pointed out that sparse and dense populations respond differently to pH. In this report I elaborate and extend these findings.

MATERIALS AND METHODS

Cell Culture and Labeling Techniques

Chick embryo cell cultures were prepared from trypsinized 10-day old Kimber Farms embryos according to a standard procedure (9, 10), and grown in medium 199 plus 2% tryptose phosphate broth and 1–5% unheated chicken serum. Except where noted, the atmosphere of the incubators consisted of 5% CO_2 in air and the temperature was 38°C. The pH was controlled by varying the amount of $NaHCO_3$ in the medium or the percentage of CO_2 in the atmosphere of the incubator. Attempts to substitute organic buffers such as hepes and tricine for bicarbonate gave irregular results in comparing low and high cell densities and had to be abandoned. Methyl-thymidine-^{3}H (New England Nuclear Corp., Boston, Mass.) labeling of DNA, extraction of nucleic acids, and radioatuographic procedures have been described (13). Cells were counted in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) after they had been suspended by trypsinization. All experiments were done in 60-mm Falcon plastic tissue culture Petri dishes (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.). Two replicate cultures were used for each experimental point. To determine the pH of the medium, tubes containing 1 ml of mineral oil were equilibrated overnight with the atmosphere of the incubator. The culture medium was drawn into a pipette and immediately discharged under the mineral oil in the equilibrated tube. The pH was determined by immersing the glass electrode of a Radiometer pH meter (Copenhagen) through the oil into the culture medium. The oil did not affect the pH reading. No correction was made for temperature.

Cell Migration

To measure the rate of cell migration, cells were scraped from a 5 mm strip down the center of a confluent sheet of cells with a rubber policeman. A scratch was made on the bottom surface of the dish to mark the border of the wound. At 24 hr, the cells were labeled with methyl-thymidine-^{3}H 16.7 Ci/mmole for 1 hr using 0.2 μCi/ml, and prepared for radioautography. The average rate of cell migration was determined over 24 hr with the aid of an ocular grid, by counting the number of cells in 10 successive strips 80 μ × 800 μ beginning at the scratch which marked the edge of the wound.

Conditional Medium

Medium of the appropriate composition and pH was incubated with confluent chick embryo cell cultures for 1–3 days. The medium was removed, floating cells were centrifuged out, and the supernatant was refrigerated until used for cell growth experiments. When used to support the growth of cells the conditioned medium was supplemented with 20% fresh medium on either the first or third day of cultivation.

Rous Sarcoma Virus Infection

The Byran strain of Rous sarcoma virus was incubated overnight with a freshly seeded culture of 10^6 chick embryo cells at a multiplicity of infection of about one. The next day the medium was replaced by an overlay of 0.5% Bacto-Difco agar (Bacto Bacteriological Products, Difco Labs, Detroit, Mich.) containing medium 199 plus 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. On day 3 when a high proportion of cells was transformed the agar overlay was removed and replaced with fluid culture medium.

Electron Microscopy

Flexible plastic cover slips (Lux Scientific Corp., Thousand Oaks, Calif.) were placed in tissue culture dishes before cells were added. The cultures were grown in the usual manner. At the apropriate time, the cover slips were removed and the cells were fixed in glutaraldehyde and osmium tetroxide. They were then embedded in Epon 812, sectioned, and stained with 25% uranyl actate (methanol) and lead citrate.

Chemicals

Diethylaminoethyl (DEAE)[1] dextran mol wt 2 × 10^6 and sodium dextran sulfate 500 mol wt 5 × 10^5 were obtained from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Stock solutions were made in glass-distilled water, and diluted directly in tissue culture medium to the desired concentration. 2-Deoxyglucose-^{3}H 6.8 Ci/mmole was obtained from New England Nuclear Corp.

RESULTS

The Effect of pH on Cell Multiplication in 5% Serum

The growth rate of chick embryo cells was studied as a function of pH between 6.9 and 8.2. In the experiment of Fig. 1, a relatively high

[1] *Abbreviations:* DEAE, diethylaminoethyl.

serum concentration of 5% was used. This causes a high rate of glycolysis and a consequent decrease with time in the pH of the medium. The initial growth rate of the sparse cultures was about the same at all pH values between 7.3 and 8.2, but the rate was reduced at pH 6.9 (Fig. 1). Cell number reached a maximum in all cultures by day 4, with the final concentration of cells roughly proportional to the initial pH from 6.7 to 8.0. The cessation of growth at the lower pH values could not have been caused by simple medium depletion, since the mere addition of $NaHCO_3$ to raise the pH to 7.6 restored rapid growth in these cultures. The cessation of growth at the highest pH, however, was due to either depletion or toxicity since the 4 day medium from these cultures did not support further growth when added to the low pH cultures.

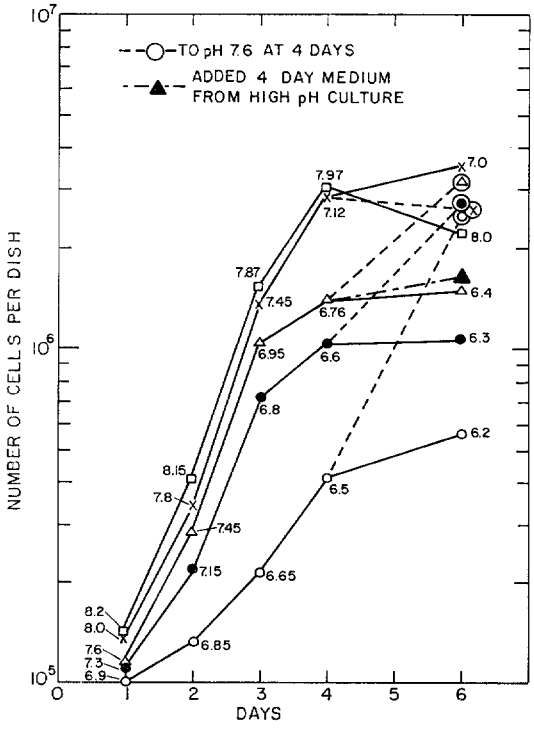


FIGURE 1 Growth rates of cells as a function of pH. Media containing 5% chicken serum and various concentrations of $NaHCO_3$ were equilibrated with the CO_2–air mixture of the incubator. 2×10^5 chick embryo cells were added to each culture. Daily cell counts and pH determinations were made. On day 4, $NaHCO_3$ was added to some of the low pH cultures to bring them to pH 7.6, and the cells were counted two days later (- - ○ - -). Also, at 4 days, medium was removed from cultures at the highest pH (then at 7.97), substituted for the medium of a culture at a lower pH (- —▲— -) and the cells were counted two days later.

pH vs. CO_2 Requirement

The observed effects on growth rate could have resulted from a dependence of growth rate either on pH or on the supply of CO_2 or HCO_3^-. To distinguish between these possibilities the effect of pH on cell growth was studied in atmospheres of approximately 5% and 0.05% CO_2 in air. Since the medium contained small amounts of buffers other than HCO_3^-, the relationship between HCO_3^-, P_{CO_2}, and pH was complex and was determined empirically. The buffering capacity of the medium in low P_{CO_2} was marginal and resulted in a sharp drop of pH in the 2 day period of growth. Despite this problem a fairly close correspondence was found between pH at the two different P_{CO_2} levels and the extent of cell multiplication (Fig. 2 *a*). When the same data were plotted as a function of the concentration of $NaHCO_3$ (Fig. 2 *b*), it can be seen that about 10 times as much $NaHCO_3$ was required to produce a given level of cell multiplication at high P_{CO_2} as at low P_{CO_2}. It was concluded that the rate of cell multiplication depended on pH and was independent of CO_2 and HCO_3^- concentrations.

pH Effects at Low Serum Concentrations

The effects of pH were studied in more detail at lower serum concentration (2%) to minimize progressive metabolic lowering of pH. Conditioned medium was used to improve the growth of low cell concentrations. Measurements were made of cell numbers and thymidine-^{3}H incorporation over a 5 day period (Fig. 3). At pH 7.5 the initial growth rate of cells was high, but a ceiling on cell numbers was reached by day 4. This does not mean, however, that growth ceased at this pH, since cells were continuously detaching from the dish and the rate of thymidine-^{3}H incorporation remained high through day 5 in the cells which remained attached.

The initial rates of growth and thymidine-^{3}H incorporation at pH 6.7 and 7.0 were 1.5–2 times lower than at pH 7.5. (It will be shown below that this difference could be eliminated by starting with an even lower cell density and using medium which had been previously conditioned for 3 days to minimize cell damage.) On day 4 the

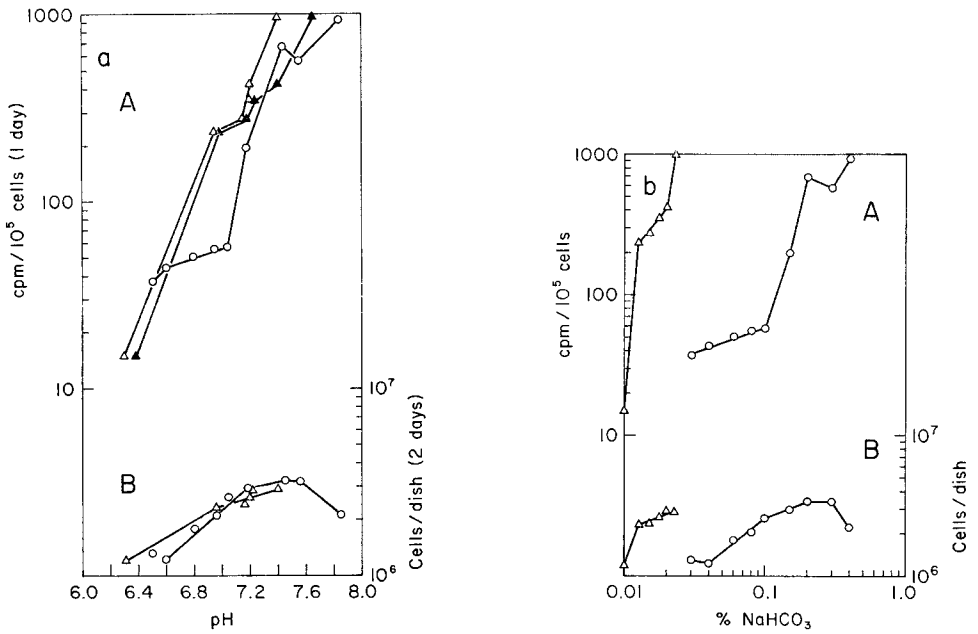


FIGURE 2 Effects on growth rate of variations in concentration of $NaHCO_3$ and P_{CO_2}. 1×10^6 cells were seeded at pH 7.4 in medium 199 containing 2% tryptose phosphate broth and 1% chicken serum. Media containing various amounts of $NaHCO_3$ were equilibrated with atmospheric air at 38°C (~0.05% CO_2) and with the atmosphere of the 38°C incubator (~5.0% CO_2). 5–10 times less $NaHCO_3$ was required to give the same pH in low P_{CO_2} of the atmosphere as that of the incubator. These media were substituted for the original medium 1 day after seeding. The following day, the pH of the medium was measured, and the rate of thymidine-^{3}H incorporation was determined. 2 days after the pH change was made, the pH of the medium was determined in the remaining dishes and the cells were counted. Fig. 2 *a* plots the results as a function of pH, and Fig. 2 *b* plots the results as a function of $NACHO_3$ concentration. Part A of each figure represents thymidine-^{3}H incorporation at one day, and part B of each figure the cell count at 2 days. ○——○, cultures in 5% CO_2; pH measured at 1 day; △——△, cultures in 0.05% CO_2; pH measured at 1 day; ▲——▲, cultures in 0.05% CO_2; pH measured at 0 days.

rates of growth and thymidine-^{3}H incorporation at these pH levels were further reduced by a factor of more than 2, although there was no metabolic reduction in pH of the medium. The addition of $NaHCO_3$ on day 4 to the pH 7.0 cultures doubled the rates of growth and thymidine-^{3}H incorporation by day 5. The pH 6.7 cultures responded to the pH increase with increased thymidine-^{3}H incorporation but not with increased cell multiplication. The latter appeared to be restricted by the accumulated damage of low pH since it was avoided (see next section) by using a more thoroughly conditioned medium.

The reduction in growth rate after day 4 at pH 7.0 occurred when the cultures were only one-half confluent. This indicates that growth inhibition can occur when no more than a fraction of the total surface of any cell is in contact with other cells.

pH Effects in Cultures Initiated at Very Low Cell Densities

A study was made of the effect of pH on the growth rate of cells at cell densities low enough to insure complete isolation of cells from one another. 2×10^4 cells were seeded per dish in conditioned medium, which was replaced the following day by media conditioned for 3 days at various pH levels. Cell counts were made daily, and the proportion of cells synthesizing DNA was determined radioautographically. At the time that a slowdown in growth began in the low pH cultures, the pH was intentionally raised in some of these cultures by adding bicarbonate, and the effect on further growth was observed. As a supplementary test for medium depletion, the medium of some cultures was transferred to newly seeded,

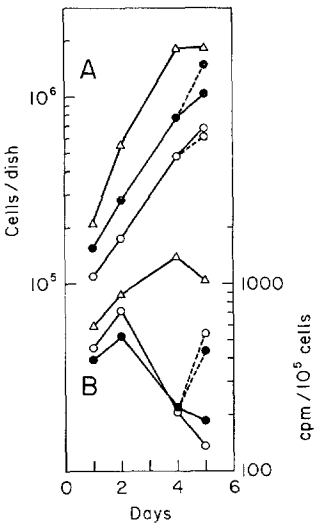


FIGURE 3 Growth and thymidine-^{3}H incorporation as a function of pH. Cultures were seeded with 2×10^5 cells at pH 7.4 in conditioned medium containing 2% tryptose phosphate broth and 2% chicken serum. At 7 hr the media were replaced with media of varying pH which had been conditioned for 1 day on growing cultures of chick embryo cells. ○——○, pH 6.7–6.8; ●——●, pH 6.9–7.1; △——△, pH 7.4–7.5. On each successive day pH, cell numbers, and thymidine-^{3}H incorporation were determined. On day 4, $NaHCO_3$ was added to some of the low pH cultures to bring them to pH 7.5, and these were assayed on the following day (– – – –). Part A represents cell numbers, and Part B thymidine-^{3}H incorporation.

low density cultures, and the effect on growth rate was measured.

There was practically no effect of pH on growth rate when the cells were at very low densities (part *A*, Fig. 4). If anything, growth at the physiological pH of 7.4, which is close to optimal for larger numbers of cells, was initially slower than at pH 6.7 and 7.0 and the cells appeared to be damaged. However, the growth rates in cultures maintained at pH 6.7 and 7.0 decreased at 5 days when these cultures had become one-third to two-thirds confluent. The low pH media at this point were fully competent to support the growth of smaller numbers of cells (part *B*, Fig. 4). The addition of bicarbonate to raise the pH was followed by a resumption of the original rapid growth rate (dashed lines part *A*, Fig. 4). Medium from the high pH cultures was partially depleted by day 5 (part *B*, Fig. 4).

The radioautographic measurements showed a sharp drop between days 4 and 5 in the proportion of cells synthesizing DNA during a 1 hr pulse of thymidine-^{3}H (part *C*, Fig. 4). Microscope examination of the cultures on day 4 showed that some cells had assumed parallel orientation in relation to one another although their borders were largely separated by some distance (Fig. 5 *a*). This pattern, indicating some type of mutual

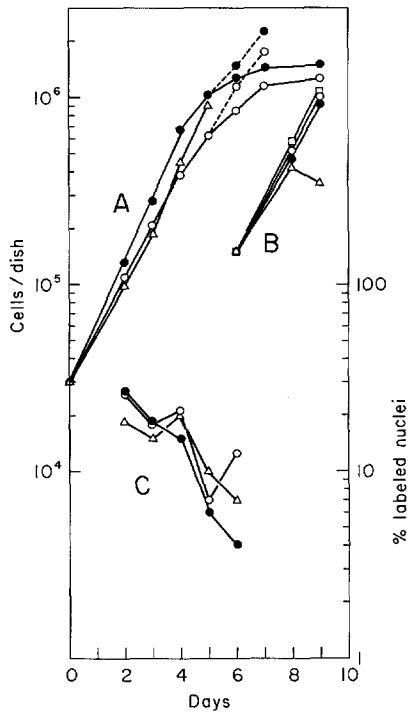


FIGURE 4 Growth, radioautography, and medium depletion in cultures initiated at very low cell density. Media containing 2% tryptose phosphate broth and 1% chicken serum at pH 6.7, 7.0, and 7.4 were conditioned for 3 days on growing cultures of chick embryo cells. Then 2×10^4 cells were seeded in conditioned medium pH 7.4. The next day, media at pH 6.7, 7.0, and 7.4 were substituted. 3 days later, 1.5 ml of fresh medium at the appropriate pH were added to the 5 ml of medium present in the culture. (A) Cells were counted daily. Broken lines indicate $NaHCO_3$ added to raise pH to 7.5 on day 5. (B) On day 6, medium was removed from some cultures in each group, adjusted to pH 7.5, and substituted for medium in fresh cultures seeded with 2×10^5 cells, to determine whether the medium was depleted. A fresh medium control (□——□) was included. (C) Thymidine-^{3}H was added to the original cultures on days 2–6 and radioautographs made (see per cent labeled nuclei on the bottom, right hand scale).

influence among the cells became dominant in the culture on day 5, but the cells were still separated by gaps distinctly visible by light microscopy along the greater length of their edges (Fig. 5 *b*). Electron-opaque material was detected in the intercellular gaps by electron microscopy (Figs. 6 *a* and 6 *b*), and may have played a role in producing the parallel orientation among cells which did not seem to be touching each other.

Direct Test of the Effect of Cell Density

A direct test of the effect of population density on response to pH was made by seeding cells at low and high densities under the same conditions and comparing the rates of thymidine-^{3}H incorporation. Thymidine-^{3}H incorporation into DNA measured 1 day after the pH shift was constant in the sparse cultures between pH 7 and 8 and only decreased when the pH was less than 7 (Figs. 7 A and 7 B). Thymidine-^{3}H incorporation in the dense cultures, however, decreased progressively as the pH was reduced below a value which varied in different experiments between 7.4 and 7.8. It is evident that the rate of thymidine-^{3}H incorporation at the higher pH levels was at least as high in the dense as in the sparse cultures.

Duration of Culture, Cell Density, and Growth Rate

A study was made of the combined effects of duration of culture and population density on the growth of chick embryo cells. Sparse and dense cultures were seeded in medium varying in pH from 6.6 to 8.0. The number of cells and the rate of thymidine-^{3}H incorporation were determined on each of 4 successive days. The pH was measured at each interval; no progressive change in pH was detected.

1 day after seeding, thymidine-^{3}H incorporation per cell in both sparse and dense cultures was insensitive to pH between pH 7.0 and 8.0 (Fig. 8). On the second day, thymidine-^{3}H incorporation in the sparse cultures was unchanged but was reduced about twofold at pH values below 7.8 in the dense cultures. On the third day, the numbers of cells in the initially sparse cultures at pH $\geq$ 7.2 had increased to about 4×10^5 per dish, and the rate of thymidine-^{3}H incorporation into DNA became sensitive to lowering pH below 7.6. The dense cultures contained more than 10^6 cells by day 3 and manifested a sharp decrease in thymidine-^{3}H incorporation with pH below 7.8. On the fourth day thymidine-^{3}H incorporation in both sets of cultures showed a pronounced sensitivity to pH. The dense cultures also displayed an abnormally high rate of thymidine-^{3}H incorporation into DNA at very high pH even when compared with sparse cultures growing at a maximum rate.

The transport experiment of Table I showed that the rate of uptake of the radioactive precursor into the acid-soluble pool increased with pH up to 7.76. It was also found that further increases of pH increased the uptake of thymidine-^{3}H even more, without increasing the proportion of labeled cells. This indicated that the extremely high levels of thymidine-^{3}H incorporation into DNA at very high pH were due to increased rates of uptake rather than to a continually increasing proportion of cells making DNA. Similarly, it was found that uridine uptake was increased at high pH. This is consistent with the demonstration that uridine uptake rises with the growth rate of chick embryo cells (21).

The effect of duration of culture on pH sensitivity is illustrated by comparing thymidine-^{3}H incorporation at pH 7.2 in the sparse and dense cultures on days 4 and 1, respectively (Fig. 8). Both sets of cultures had about 5×10^5 cells at these times, but the initially sparse cultures which had grown for 4 days incorporated thymidine-^{3}H at about one-fourth the rate of the initially dense cultures which had grown for only 1 day. This result suggests that cells become increasingly sensitive to inhibition by lowered pH with each successive day in culture. This is also suggested by the decreasing rate of DNA synthesis between days 3 and 4 despite the constancy of cell numbers. The possibility cannot be excluded, however, that effects are caused by partial depletion of the medium.

pH, Cell Migration, and Cell Growth

Effects of pH on both cell migration and DNA synthesis were studied by removing a strip of cells from a confluent sheet with a rubber policeman. Cells along the edge of the wound migrated into the denuded area. There they constituted a locally sparse population in contrast to the dense population of the confluent sheet of the undisturbed portion of the culture, enabling a study of pH effects on DNA synthesis among sparse and

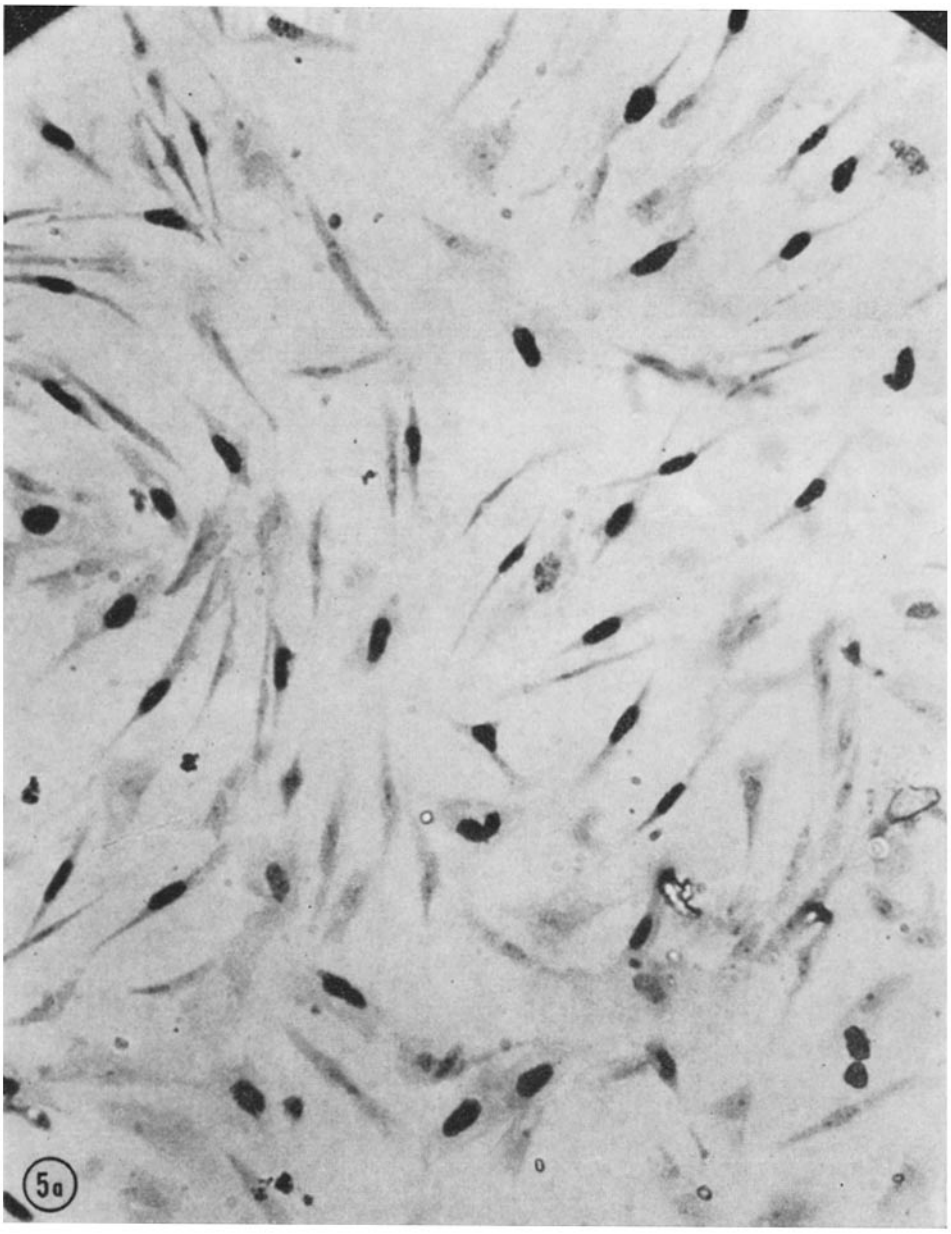
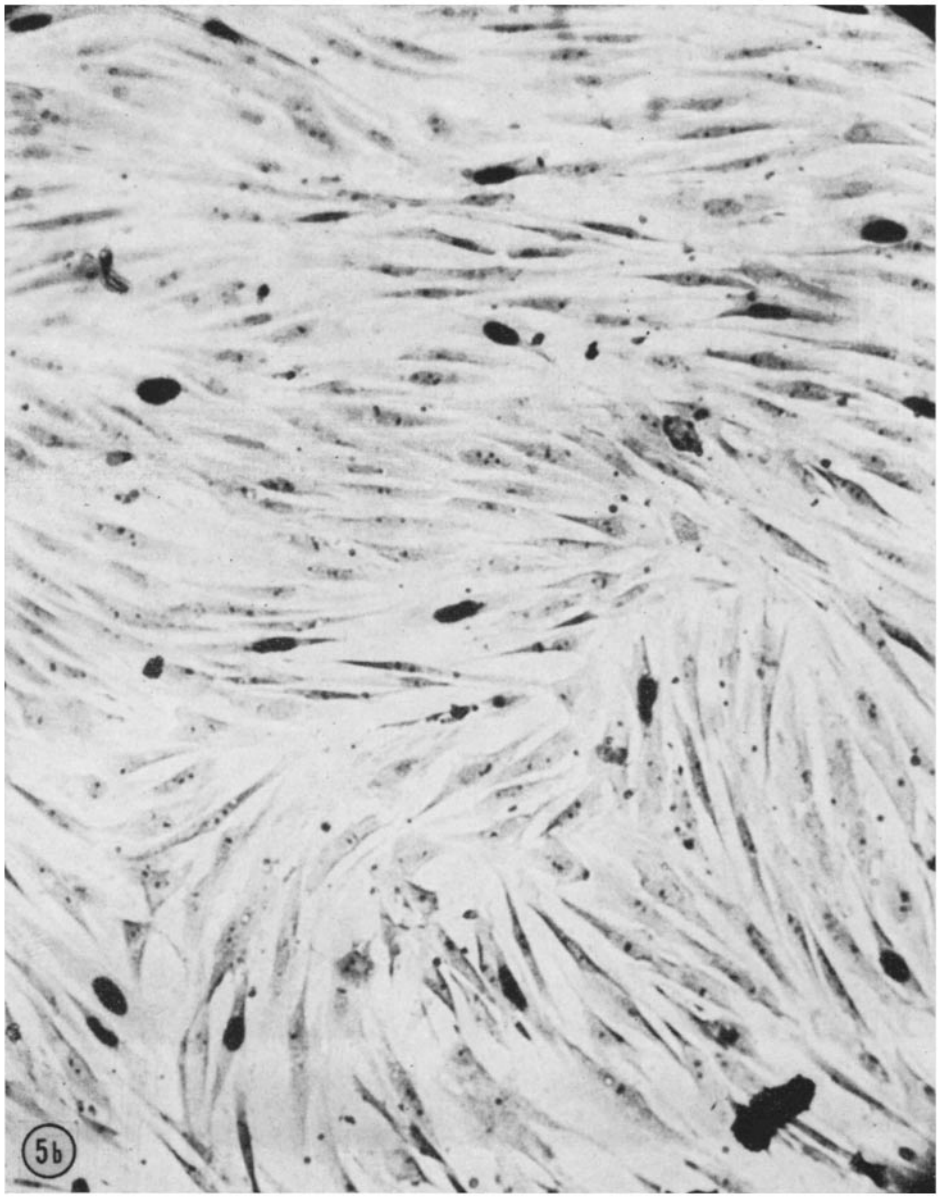


FIGURE 5 Radioautographs of cells grown at pH 6.7 in Fig. 4 on day 4 (Fig. 5 *a*) and day 5 (Fig. 5 *b*) of cultivation. See legend to Fig. 4. × 570.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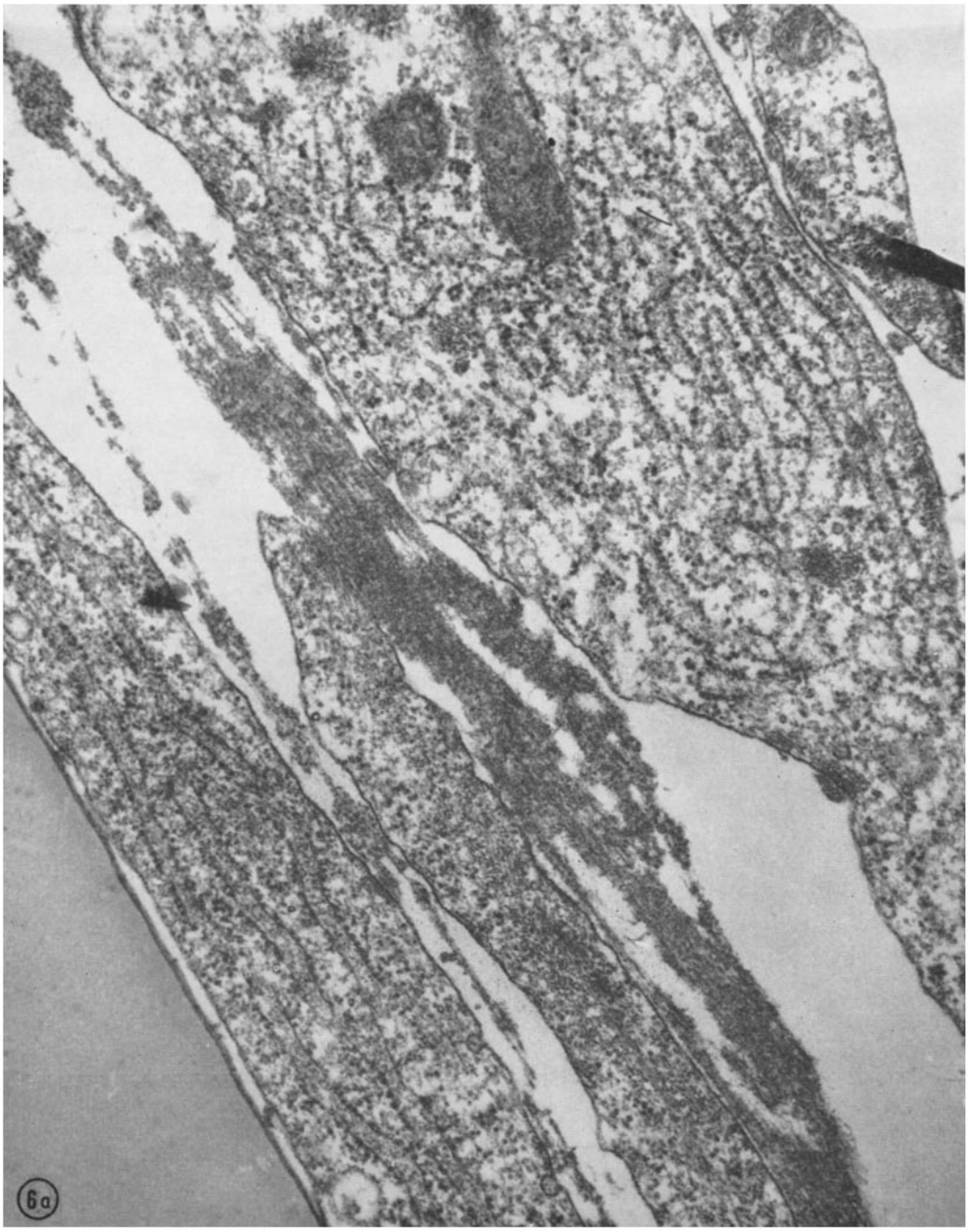
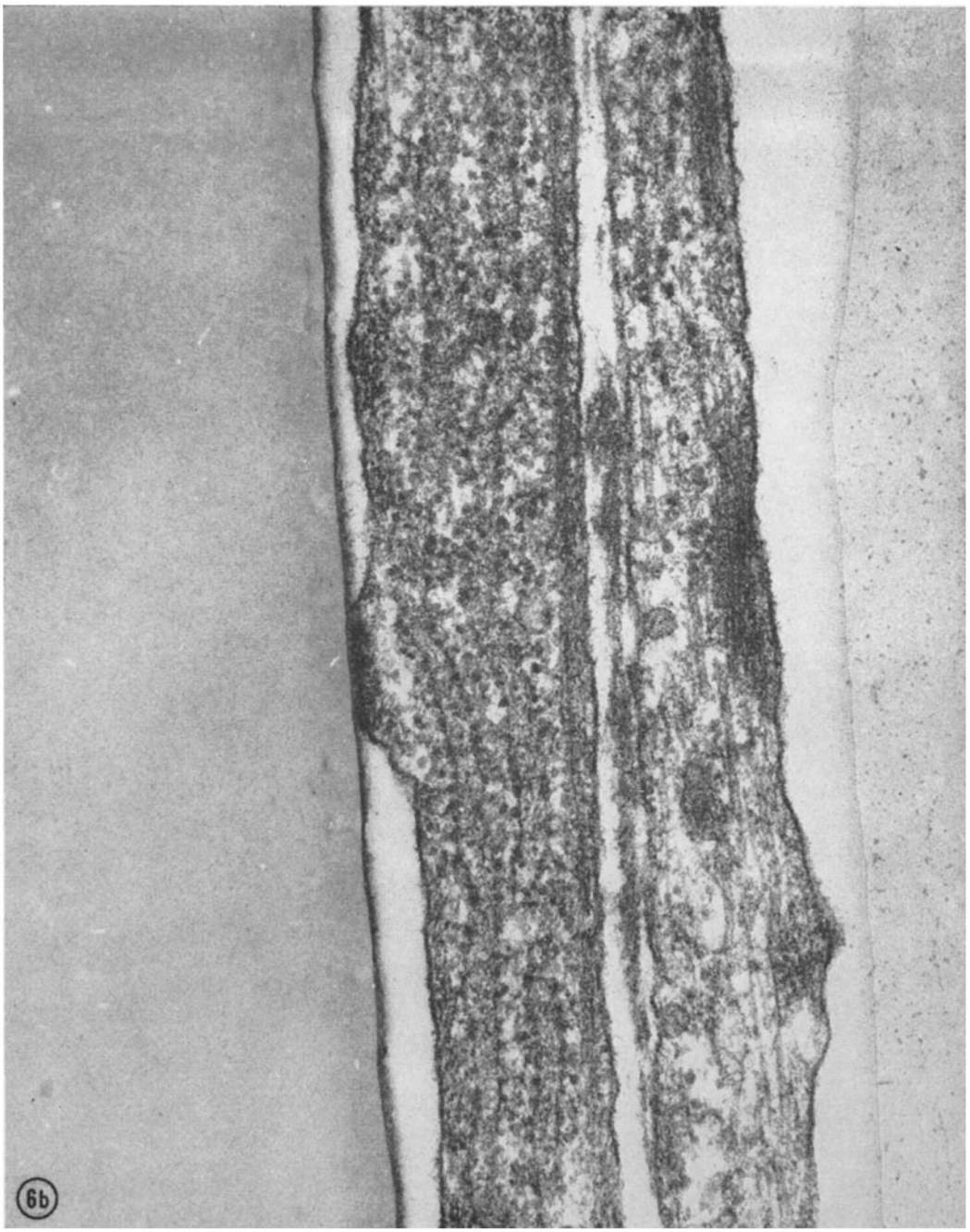


FIGURE 6 Electron micrographs of cells grown at pH 6.7 (Fig. 6 *a*) and pH 7.4 (Fig. 6 *b*). 1×10^6 cells were grown on plastic cover slips for 3 days at the indicated pH in medium containing 2% tryptose phosphate broth and 2% chicken serum. Cells were fixed *in situ* and sectioned as described in Materials and Methods. × 73,500.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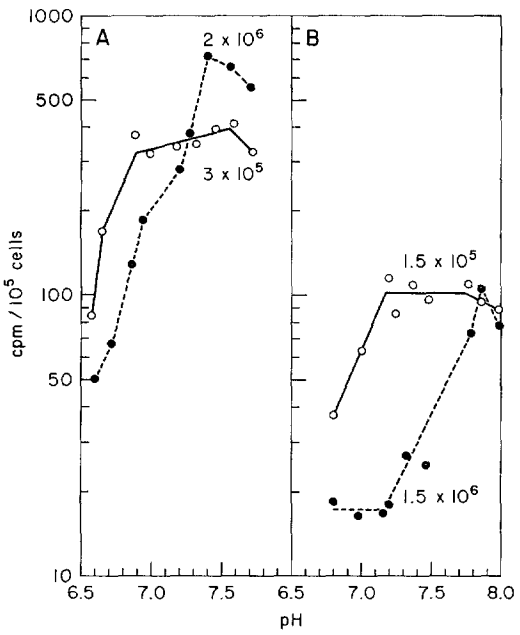

FIGURE 7 Thymidine-^{3}H incorporation as a function of pH in cells seeded at low and high densities. Cells were seeded at 1×10^5 and 1×10^6 per dish in fresh medium containing 2% tryptose phosphate broth and 2% chicken serum. The next day, the medium was changed to equilibrated media with varied pH. The following day, cell counts were made (approximate numbers shown on the curves) and the rates of thymidine-^{3}H incorporation were measured. Fig. 7 *A* and 7 *B* represent two separate experiments.

dense populations in the same dish. At 24 hr the cells were exposed for 1 hr to 4 μCi/ml of thymidine-^{3}H, and prepared for radioautography. The number of cells which had migrated into the denuded area as well as the proportions of labeled nuclei in the denuded and confluent areas were determined. The rate of migration of cells into the denuded area was sharply reduced by lowering the pH of the medium, as was the proportion of cells synthesizing DNA in the confluent regions (Fig. 9). The proportion of cells synthesizing DNA in the denuded region, however, was high in all cases, and independent of pH. This indicates that the rate of cell movement decreases with decreasing pH but that those cells which do move into the wound at low pH synthesize DNA at a maximal rate.

pH Effects on the Growth of Malignant Cells

A comparison was made of the effect of pH on the growth rates of normal and malignant cells. To this end, cells were infected with Rous sarcoma virus and overlaid with agar medium to maximize the transformation. The rates of thymidine-^{3}H incorporation were measured in dense populations of transformed and nontransformed cells, and in sparse populations of nontransformed cells. The nontransformed populations were also overlaid with agar. It was found that the overlay with agar distorted the response of the nontransformed cells to pH, so that the crowded cells did not incorporate thymidine-^{3}H at a high rate even at high pH (Fig. 10). Between pH 6.8 and 7.4 the crowded Rous sarcoma culture behaved more like sparse normal cultures than like crowded normal cultures. This was not the case at pH > 7.4, and the discrepancy remains unresolved. It should be noted, however, that the Rous-infected cultures contained a moderate proportion of normal-looking cells which might have influenced the response to pH.

pH and Cell Permeability

Small but definite increases in the rate of transport of thymidine (Table I) into dense cultures were found with increasing pH. A much more marked effect of pH was observed on the transport of 2-deoxyglucose-^{3}H into dense cultures is shown in Fig. 11. Only a small effect of pH on transport of 2-deoxyglucose was observed in sparse cultures. The increased rate of 2-deoxyglucose transport with pH in dense cultures was of the same magnitude as the increased rate of thymidine-^{3}H incorporation into DNA.

Polyelectrolyte Effects on pH-inhibited Cells

There are indications that the pH at the surface of the cell is lower than that of the bulk medium (4). This differential presumably arises from the association of protons with the fixed negative changes of the cell surface. The adsorption of cationic polymers such as DEAE-dextran to the cell surface would be expected to increase the local pH, and the adsorption of anionic polymers such as dextran sulfate should decrease it, without necessarily changing the pH of the bulk medium. Accordingly, DEAE-dextran and dextran sulfate were added to cells in media of differing pH. DEAE-dextran stimulated the incorporation of thymidine-^{3}H into DNA when it was added in low concentrations to confluent cells inhibited by low pH (Fig. 12). The concentration of DEAE-

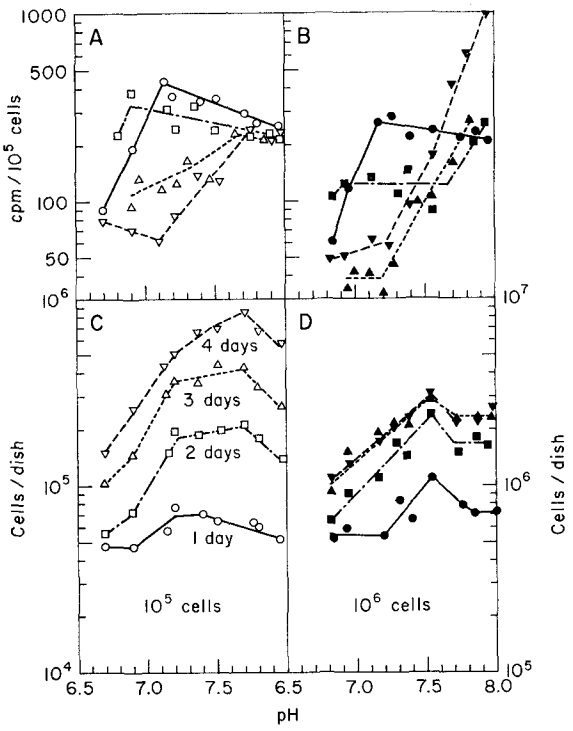

FIGURE 8 Cell density, duration of culture, and pH effects on the growth of cultures at various pH levels. 1×10^5 and 1×10^6 cells were seeded in fresh medium with 2% tryptose phosphate broth and 2% chicken serum at pH 6.6–8.0 Each day for 4 days, pH, cell number, and thymidine-^{3}H incorporation rate were determined. The top two panels (A and B) represent thymidine-^{3}H incorporation, and the bottom two panels (C and D) cell numbers.

10^5 cells	Day	10^6 cells
○—○	1	●—●
□—·—□	2	■—·—■
△— — —△	3	▲— — —▲
▽— — —▽	4	▼— — —▼

dextran required to stimulate DNA synthesis varied with cell population density. Higher concentrations of DEAE-dextran were toxic to the cells. Dextran-sulfate inhibited DNA synthesis, at all doses which had any biological effect (Table II).

DISCUSSION

The response of chick embryo cells to pH has been shown to be very much a function of the population density of the cells. When the population density is low, the growth rate of the cells is decreased only when the pH is reduced below neutrality. Even this slowdown may be a spurious effect of the toxicity of acid pH to low concentrations of cells, since the use of conditioned medium which enhances the growth of low concentrations of cells (11) permits the growth of sparse cultures at almost as rapid a rate at pH 6.7 as at a neutral or higher pH. Reductions in pH below 6.7 are not tolerated by sparse cultures for more than a few hours.

When the population density is high, the rates of cell multiplication and of thymidine-^{3}H incorporation into DNA are markedly dependent upon pH. In general, both parameters increase up to a pH of about 7.6. Further increases in pH may have no effect on the rate of cell multiplication, or may even be inhibitory to cell multiplication (Fig. 8). The rate of thymidine-^{3}H incorporation into DNA, however, may increase with pH up to and above 8.0. This is particularly true in

TABLE I

pH versus Uptake of Thymidine-^3H into Acid-Soluble and Acid-Insoluble Fractions

	cpm/10^5 cells in cultures initially seeded with			
	1×10^5 cells		1×10^6 cells	
pH	Pool	DNA	Pool	DNA
6.6	84	226	25	63
6.78	87	200	38	110
7.35	74	170	48	197
7.76	95	195	56	346

Two groups of cultures were prepared with 1×10^5 and 1×10^6 cells in medium 199 plus 2% tryptose phosphate broth and 1% chicken serum. After 1 day of culture, the medium was replaced by one of the same composition but varying in pH. On the following day, the cells were exposed to 0.2 μCi/ml thymidine-^3H for 1 hr. They were washed and extracted with 5% trichloroacetic acid at 5°C for 5 min to obtain the acid-soluble pool. They were then exposed to 10% trichloroacetic acid at 70°C for 2 hr to hydrolyze the DNA, and the radioactivity of both samples was measured in the scintillation counter. Sister cultures at each pH were trypsinized, and the number of cells was determined in the Coulter counter.

very crowded cultures. The failure of the increase in cell number to keep pace with the increased rate of thymidine-^3H incorporation at very high pH is partly caused by cell death and detachment. There is also evidence that the increased incorporation of thymidine-^3H at high pH does not accurately reflect increased DNA synthesis, since the proportion of cells synthesizing DNA as measured by radioautography is not as high as expected from the over-all increase in thymidine-^3H incorporation in hot trichloroacetic acid extracts of the cells. The discrepancy between these two measurements is due in large measure to an increase with pH in the rate of transport of thymidine-^3H into the cell.

The cell counts and radioautographic measurements leave no doubt, however, that cell multiplication and DNA synthesis proceed at a relatively rapid rate in dense cultures maintained at high pH. In fact with a fresh change of medium the phenomenon of density-dependent inhibition of cell multiplication is consistently apparent only at pH less than 7.4 (Fig. 3). At the lower pH levels it is certain that population density plays an important part in limiting the rate of cell multiplication. The particular population density at which growth slows down is determined by the pH of the culture. At very high pH the restrictions on growth rate are set by depletion of nutrients

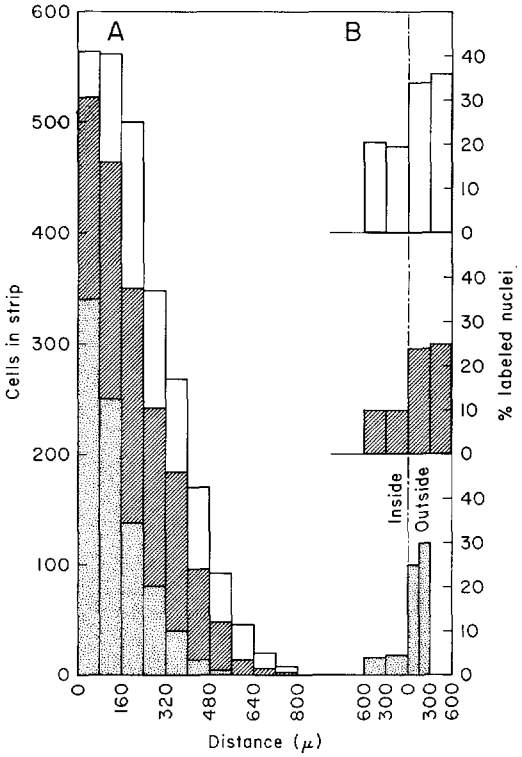

FIGURE 9 Cell movement and thymidine-^3H incorporation as functions of pH. 1×10^6 cells were seeded in medium containing 2% tryptose phosphate broth and 1% chicken serum. At 2 days, the medium was replaced by media at pH 6.6 ⊡; pH 7.0 ▨; and pH 7.5 □. The following day a strip of cells was removed with a rubber policeman, and a scratch was made on the underside of the dish to mark one border of the denuded strip. 24 hr later, thymidine-^3H was added for 1 hr, and the cells were prepared for radioautography. Part A, left side. When the radioautographs were developed, the migration of cells from the border of the scratch was measured with a 1 cm^2 ocular grid, at × 125 magnification. Cells were counted in 10 successive bands 80 μ wide beginning at the border and extending into the denuded region. The ordinate of part A represents total of cells for 10 different fields in each band. Part B, right side. Subsequently the magnification was increased to × 300 and the proportion of cells with labeled nuclei was determined as a function of distance from the edge of the scratch. The proportions were determined in the direction of the confluent sheet (inside) and in the direction of the denuded region (outside).

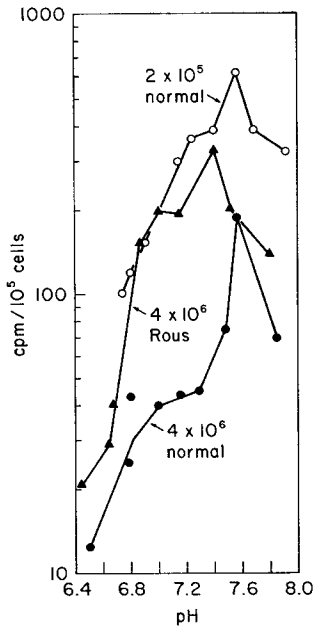

FIGURE 10 Effect of pH on thymidine-^{3}H incorporation by Rous sarcoma cells. One set of cultures was prepared with 1×10^5 cells, and two sets with 1×10^6 cells. One of the latter sets was infected with Rous sarcoma virus. The next day, the cultures were overlaid with 0.4% agar containing 5% tryptose phosphate broth, 2.5% calf serum, and 1% chicken serum, at various pH levels. 2 days later the agar was removed, cells were counted in some cultures and thymidine-^{3}H incorporation was measured in others.

in the medium, and the toxic effects of high pH. At very low pH the limiting effects of population density can be detected before the culture becomes confluent. Examination of the culture at this time by light and electron microscopy reveals little, if any, evidence of direct contact between the plasma membranes of the cells. Indeed the cells are separated by distances of greater than 150 A along most of their borders. Aside from a decreased growth rate, however, there is evidence for mutual cellular interaction in the parallel orientation of the cells. This suggests that some intercellular material is present which is not readily detected by the microscope techniques commonly employed. Indeed, electron microscopy does reveal fragmentary material of high electron density in some of the intercellular spaces (Fig. 6). The fragmentary distribution of the material suggests that some of it is removed during fixation and embedding. The nature of this intercellular material is as yet unknown. It does not have the characteristic spacing of collagen. However, the ability of proteolytic enzymes to detach cells from one another, and to stimulate the growth of density-inhibited cells even without detaching them (16), suggests that the intercellular material contains protein. It might be related to the macromolecular conditioning factor released by cells, which supports the survival and growth of cells at very low population densities (9, 11). Since glycoproteins have been identified as major constituents of cell surface coats (23), it will be of interest to determine whether their properties vary with pH.

pH was also shown to affect the rate of cell migration away from the dense regions of a culture in a manner similar to its effect on multiplication of cells in the dense regions. At the same time it was shown that those cells which did migrate multiplied at a high rate even at very low pH. This result is of course complementary to that obtained with sparse populations in conditioned medium. It resuscitates the question about the relationship between cell movement

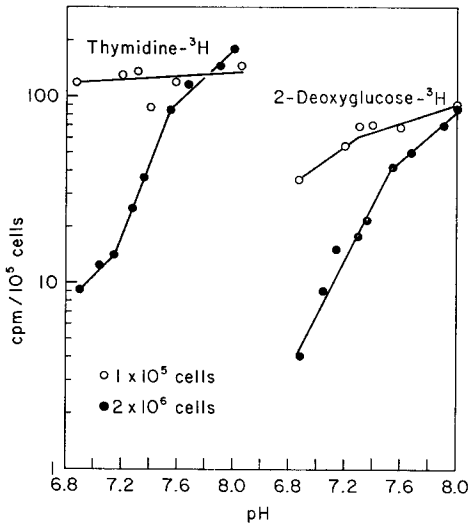

FIGURE 11 Comparative effects of pH on the uptake of 2-deoxyglucose-^{3}H and the incorporation of thymidine-^{3}H. 1×10^5 and 2×10^6 cells were seeded in medium with 2% tryptose phosphate broth and 1% chicken serum. The next day, they were switched to media of pH varying from 6.6 to 8.0. The following day, the rates of incorporation of 2-deoxyglucose-^{3}H into acid-soluble, and of thymidine-^{3}H into acid-insoluble, materials were determined in separate cultures for each pH. O——O, 1×10^5 cells; ●——●, 2×10^6 cells.

and cell growth. Certainly, the growth rate of all cells is not tied to the extent of lateral displacement of the cells, since malignant cells, whose growth rates are much less dependent than normal cells on population density, migrate at a lower rate in culture than do normal cells (1, 2, 20). The membrane movements of the malignant cells consist of the elaboration of random, spiky processes in contrast to the coordinated, undulating movements of the leading lamella of normal cells (1). Thus, membrane motility produces sustained directional movement of normal cells but not of malignant cells. It seems likely, therefore, that it is membrane activity rather than distance traveled which is related to cell growth. In this regard it is of interest to note that pH has been found to affect membrane motility (19, 22) in a manner similar to its effect on growth and movement.

Further evidence of cell surface change with pH is afforded by the altered permeability of cells with pH. The permeability of chick embryo cells to 2-deoxyglucose, an analog of glucose, is dramatically affected by pH over a 24 hr period. Neuronal excitability, which depends on transmembrane exchange of ions, is similarly dependent on pH (18), as is the exchange of sodium by red blood cells (5) and of chloride by muscle cells (7). It seems likely that altered permeability to ions and small molecules plays an important and perhaps primary role in shaping the growth response of cells to alterations in pH and cell population density.

There remains the question of why pH affects dense populations more than it does sparse populations. Membrane motility in cells is most prominent along their free edges (1), and it is greatly reduced in cells surrounded by other cells. It is possible that the combined restrictions on membrane motility of low pH and cell-cell association are required to inhibit the growth of the cells. This is equivalent to saying that isolated cells at physiological pH have an excess of membrane activity (permeability?), and that the reduction caused by low pH is without consequence on growth rate. Some support for this suggestion is derived from the observation that the optimal pH for the growth of very sparse cells (Fig. 4) is about 7.0 whereas it is about 7.6 for crowded cells (Fig. 8). I interpret this to mean that isolated cells are already optimally active and permeable at neutral pH and are damaged by the stimulatory effects of higher pH. It is also to be noted that isolated cells have a larger over-all surface area available for the passage of molecules from the medium than do normal cells.

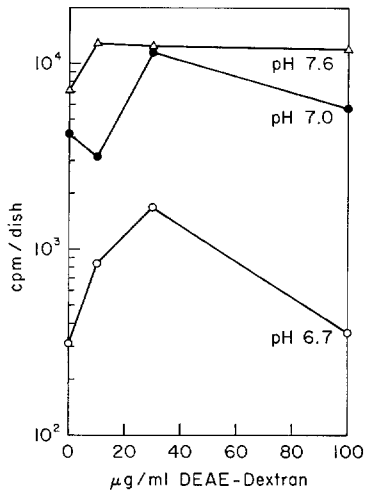

FIGURE 12 Polyelectrolyte effects on thymidine-^{3}H incorporation in cells at different pH levels. 1×10^6 cells were seeded in medium containing 2% tryptose phosphate broth and 1% chicken serum, and incubated for 3 days. Media at pH 6.7, 7.0, and 7.4 were then substituted. The next day, 0, 10, 30, and 100 μg/ml of DEAE-dextran were added. On the following day, the rates of thymidine-^{3}H incorporation were determined.

TABLE II

Dextran Sulfate vs Thymidine-^{3}H Incorporation

	cpm/10^5 cells in cultures at pH		
Dextran sulfate	6.5	6.8	7.0
μg/ml			
0	70	671	1630
1	80	805	1600
2	60	374	814
5	37	195	455

1×10^6 cells were seeded in medium containing 2% tryptose phosphate broth and 1% chicken serum and incubated for 3 days. Media at a range of low pH values were substituted, and 6 hr later the indicated concentrations of dextran sulfate were added. The following day the rate of thymidine-^{3}H incorporation was determined.

Ceccarini and Eagle (3) recently described the variation with pH in growth rates of several cell lines, and concluded that "early contact inhibition

is frequently not observed" when fluctuations in pH are avoided by using nonvolatile buffers. This statement is not borne out by the data presented in their paper. In Fig. 4 of that paper it may be seen that the day on which the growth rate of each cell line decreases is independent of the buffer used, and largely independent even of pH. The cell concentration at which the slowdown occurs is dependent on the buffer and pH but the effects of each cannot be distinguished from the other because of the cited fluctuations in HCO_3^- buffer. From the data presented, it can only be concluded that density-dependent inhibition of growth occurs under conditions of either fluctuating or stable pH. In my experiments, pH fluctuations in HCO_3^--buffered medium were minimized by careful CO_2 control, and the use of low serum concentrations, which avoids the excessive uptake of glucose and consequent production of lactic acid.

It is of considerable interest that Ceccarini and Eagle found little evidence of differential pH response in the first 3–6 days of culture (Fig. 3 of reference 3) when the cell densities were low. This is in agreement with my conclusion that the pH effects on growth are most marked at higher cell densities. It is of further interest that they found (Table I of reference 3) that diploid cell lines and those aneuploid lines notably subject to density-dependent inhibition, i.e. 3T3, have growth optima at pH 7.5–7.8, while most of the other aneuploid and transformed lines have considerably lower pH optima. These data lend support to my finding that crowded Rous sarcoma cells are less subject to growth inhibition than are the crowded normal cells from which they derive. Unfortunately, no firm conclusion can be made about the generality of this finding until a wider variety of normal cells and their matched malignant derivatives are compared.

The relative insensitivity of Rous sarcoma cells at high population density to pH is consistent with their generally recognized insensitivity to density-dependent growth inhibition (15) and to contact inhibition of locomotion. In effect, crowded Rous sarcoma cells behave like isolated normal cells. This may be the result of the lowered adhesiveness of malignant cells (1, 6), which allows them a high degree of membrane activity even when surrounded by other cells. The corollary of this suggestion is that the restraining effect of cell density on the growth rate of normal cells in culture arises from restricted membrane activity. Since the greatest activity of cell membranes in culture is at their free edges (1), even limited tangential contact with other cells on the same surface might have a profound effect on membrane activity, and consequently on other membrane-dependent cell activities.

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