Lawrence Berkeley National Laboratory
Recent Work

Title

DEPENDENCE OF THE DIFFERENTIATED STATE ON THE CELLULAR ENVIRONMENT: MODULATION OF COLLAGEN SYNTHESIS IN TENDON CELLS (ASCORBATE/CELL DENSITY/ CELL CULTURE)

Permalink

https://escholarship.org/uc/item/48f033mk

Author

Schwarz, R.I.

Publication Date

1977-07-01

eScholarship.org Powered by the California Digital Library
University of California

U U U U 4 8 U 2 9 3 3

UC-48 c.1

Submitted to the Proceedings of the
National Academy of Sciences

LBL-6494
Preprint

DEPENDENCE OF THE DIFFERENTIATED STATE ON THE CELLULAR ENVIRONMENT: MODULATION OF COLLAGEN SYNTHESIS IN TENDON CELLS (ASCORBATE/CELL DENSITY/CELL CULTURE)

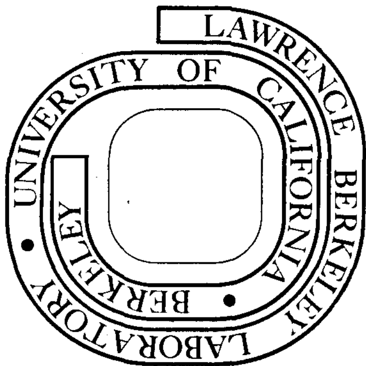
Richard I. Schwarz and Mina J. Bissell

July 6, 1977

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48

For Reference

Not to be taken from this room



RECEIVED
LAWRENCE
BERKELEY LABORATORY

OCT 17 1977

LIBRARY AND
DOCUMENTS SECTION

LBL-6494 c.1

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

0 0 J 0 4 8 0 2 9 3 6

DEPENDENCE OF THE DIFFERENTIATED STATE ON THE CELLULAR ENVIRONMENT: MODULATION OF COLLAGEN SYNTHESIS IN TENDON CELLS

(ascorbate/cell density/cell culture)

Richard I. Schwarz and Mina J. Bissell

Laboratory of Chemical Biodynamics

Lawrence Berkeley Laboratory

University of California

Berkeley, California 94720

0 0 0 0 4 8 0 2 9 3 7

ABSTRACT

Primary avian tendon (PAT) cells, if placed in an adequate environment, are capable of retaining both the full expression of differentiated function and a correct morphological orientation for one week in culture. At high density and in the presence of ascorbate, PAT cells are fully stabilized in that they devote 25-30% of their total protein synthesis to collagen, a level comparable to tendon cells *in ovo*. However, cells at either low density or in medium without ascorbate, synthesize collagen at only a third of this level. If plated on a collagen matrix, PAT cells will orient themselves in a manner similar to tendon cells *in vivo*. Furthermore, the cells are capable of fully modulating the percent collagen synthesis with addition or removal of ascorbate and serum. The variation in the percentage of collagen produced is a result of alterations in collagen synthesis rather than changes in total protein synthesis or hydroxylation of proline in collagen. PAT cells, therefore, provide a suitable model for understanding the stability of the differentiated state, the mechanism of action of ascorbate, and the regulation of collagen biosynthesis.

INTRODUCTION

Understanding what regulates gene expression at a cellular and molecular level requires a rigorously defined and controlled environment. Despite efforts of the cell biologists for the past 70 years, most cells when removed from the organism and placed in culture, lose their ability to remain differentiated (1-5). One conclusion that can be drawn from this universal phenomenon is that very few, if any, tissue specific functions are "constitutive". That is, despite the fact that synthesis of differentiated products is stable *in vivo*, its continued expression relies on factors that are no longer present in culture. To study function when cells are placed in culture, two approaches are therefore possible. One is to allow the cells to adapt to and reach an equilibrium with their new environment with invariable loss of qualitative and quantitative expression of function. This is the approach traditionally taken in the past. Alternatively the culture environment can be modified to maintain the differentiated state of the cell. This latter approach, initiated by Schwarz *et al*., (5,6) for avain tendon cells, has been pursued in this research in order to achieve a culture system which more closely resembles the *in vivo* state of cells in terms of collagen biosynthesis.

The previous research on collagen synthesis using "fibroblasts" that had adapted to standard cell culture conditions has been confusing for several reasons. First, most cells in the body have the capability to synthesize and secrete some collagen; it is the type and the quantity that varies (7-9). Therefore, the level of differentiation retained by the cell in culture can only be delineated with a knowledge of the *in vivo* origin of the fibroblast. This information is unavailable for almost all cell lines used currently. Second, in the early research on collagen synthesis (continued on page 3)

0 0 0 0 4 8 0 2 9 3 8

by fibroblasts in culture, an assay was used which did not distinguish between increased percentage of collagen synthesis and increased activity of prolyl hydroxylase, the enzyme involved in hydroxylation of proline in collagen (10,11). This assay relied on determining the ratio of hydroxyproline (which is almost exclusive to collagen) to total proline incorporated as a measure of collagen synthesis. The role of factors, such as lactate and ascorbate which could affect collagen synthesis by changing either the degree of hydroxylation of proline and/or the percentage of collagen synthesized could not be adequately described using this assay (11). Third, while a more recent assay for collagen synthesis is independent of the degree of hydroxylation (12) the cell lines utilized are not stable with time in their ability to either produce collagen or to respond to such factors as lactate and ascorbate. Cell lines, 3T3 and 3T6, which are the most frequently used culture systems for research on collagen synthesis, have largely lost their ability to synthesize collagen (10,11, 13-16). Fourth, Schwarz *et al*., (5) using PAT cells, have shown that serum levels in excess of 0.5% is inhibitory to the percentage of collagen synthesized. Since almost all earlier research used serum in excess of this level, the intrepertation of the results become even more difficult. It is therefore reasonable to question whether the cell systems used in previous studies of collagen biosynthesis in culture adequately reflect the *in vivo* cell, a point also raised by Peterkofsky and Prather (16).

To avoid these problems, we have used PAT cells which are known to produce 25-30% collagen before being placed in cell culture (5,7); we have grown them at low serum levels (except where we want to test the action of serum); and we have used an improved assay for collagen (5,12). In this paper, we explore the complex interaction between the cell and its environ-

ment which results in the stabilization of differentiated function and correct morphological orientation. We show that under appropriate conditions loss of function need not occur when cells are placed in culture. We further show that when functional loss does occur, it is a phenotypic response of the cell to its environment in that it is fully reversible when conditions are corrected.

MATERIALS AND METHODS

Isolation of Cells

Tendon cells were isolated from 16-day chick embryos as previously described, (5-7) with small modifications as follows: To slow down the rate of tissue dissociation, trypsin was reduced to 0.15%, serum was raised to 5%, and the number of tendons per ml of solution was increased. The optimum time for dissociation was found to be around 60 minutes.

Cell Culture and Media

Cells were seeded at 1.2×10^6 cells/flask (Falcon, 25 cm^2) in 5 ml of F12 medium (17) (Gibco) with 0.1 g/l streptomycin sulfate and 10^5 units/l penicillin, without serum. After approximately 45 minutes, when the cells had attached, 10 ml of fresh medium with 0.5% fetal calf serum (Gibco; deactivated at 56 C for 1/2 hr) was added. In lactate containing medium, sodium lactate (Gibco) was included in the preparation. Because ascorbate is unstable in culture medium (11), a 100x stock solution of sodium ascorbate (Gibco) was freshly prepared every other day for addition to the medium. Culture medium was changed daily. Variations from the above procedures are described in the figure legends.

Collagen Assay

Cells were pulsed with 1 ml of medium containing 50 uC of ^3H proline (2'-3'-^3H, New England Nuclear) for 3 hr. The pulse was stopped by addition

0 0 0 0 4 8 0 2 9 3 9

of 5 M NaOH to a final concentration of 0.25 M. Collagen was assayed by a modification of a collagenase method (12) as previously described (5). The percentage of collagen made on day "0", the day of isolation, was found to be very reproducible. As a result, day "0" assays were not performed for every experiment, and instead an average point derived from previous experiments (six) was used (76% ± 3.3).

Cell Counts

Cells were removed from flask with trypsin (0.05%) and were gently pipetted to eliminate clumps. They were then counted in a coulter counter.

Collagen Coated Flasks

Purified acid soluble collagen (Calbiochem; 5 mg/ml) was dissolved in 0.5 M acetic acid. The solution (.2 ml) was spread over the surface of the flasks. The collagen was precipitated by addition of 3 mls of 20% (w/v) NaCl and fixed by the addition of 0.1 ml of 2% glutaraldehyde (Poly Science Co.). After 6 hr the flasks were rinsed with saline, and 10 mg of bovine serum albumin (Sigma) was added to inactivate any remaining glutaraldehyde. After incubating at 38 C for 12 hrs, the flasks were rinsed several times with saline and used as described above except that cells were allowed to attach in the medium containing serum.

RESULTS

Stabilization of Differentiation by Ascorbate and Density

The major role of ascorbate on collagen synthesis appears to be reversed when cultured cells are compared to *in vivo* cells. In the body ascorbate deficiency leads to a marked decrease in the ability of cells to make sufficient collagen (18). To a lesser extent ascorbate appears to affect the hydroxylation of proline in collagen. In late passage cell lines in culture, ascorbate only influences the degree of hydroxylation and not the percentage of synthesis (11,14). In early passage cells, ascorbate

does affect the percentage of collagen synthesis, but only to a small degree (19). The inability of the culture cell to reflect the *in vivo* situation could stem from the fact that the conditions previously employed for cell growth tend to inhibit not only the percentage of collagen synthesized, but also the ability of the cell to respond correctly to environmental stimuli. Therefore, a primary cell grown in medium which did not inhibit the expression of collagen, might respond to ascorbate as cells do *in vivo* by increasing the synthesis of collagen. Therefore, the ability of ascorbate to affect the level of collagen synthesis in PAT cells was tested. Furthermore, since it was already demonstrated that percent collagen synthesis in PAT cells is sensitive to lactate addition (5), a comparison of ascorbate and lactate effects was also performed.

PAT cells were grown in F12 with the following four additions: 1) 0.5% serum 2) 0.5% serum + 50 μg/ml sodium ascorbate 3) 0.5% serum + 37 mM sodium lactate the NaCl concentration was reduced by 37 mM in order to maintain a constant Na^+ concentration; this resulted in a better cell growth. 4) 0.5% serum + 50 ug/ml sodium ascorbate and 37 mM sodium lactate the NaCl concentration was reduced by 37 mM . The cells were assayed for percent collagen synthesis at several points during the week. The data (Fig. 1A) may be grouped into two distinct periods corresponding to the beginning and the end of the week. As the cells adapted to culture conditions, there was a rapid, 2-3 fold drop in the percentage of collagen synthesized. This was independent of the addition of either ascorbate or lactate. At the end of the week, the cells were sensitive to the medium components, responding most strongly to ascorbate. By day 6, the collagen synthesis in the ascorbate containing medium was completely restored to the original level (30% in Fig. 1A). The data also indicated that ascorbate and lactate effects were not synergistic, i.e. the addition of both com-

0 0 0 0 4 8 0 2 9 4 0

pounds was no more effective than ascorbate alone. However, since even in the absence of added lactate, cells excrete this compound into the medium (19) its role as a necessary cofactor cannot be ruled out.

The second period, corresponding to the end of the week, is also a time when the cells are crowded together and thus grow more slowly. To clarify whether or not the slow rate of growth was responsible for increased collagen synthesis, cell number was determined simultaneously with collagen synthesis (Fig. 1B). PAT cells propagated rapidly with and without ascorbate. The generation time increased from 1 to 2 days as the cells approached a high density. It may be deduced from the data that the similarity in the growth rates did not correlate with the widely differing percentages of collagen synthesized. In addition, the slow generation time of cells in medium containing lactate was not beneficial to collagen production. This agrees with the previous reported results that the slower growth caused by removal of serum did not affect the percentage of collagen synthesized (5). Therefore, a shift down in the rate of cell growth by itself does not promote a high percentage of collagen synthesis.

It is obvious from Fig. 1A that PAT cells respond very differently to ascorbate at the beginning and at the end of the week. The simplest hypotheses to explain this fact is that the higher density attained by the end of the week plays a role in the modulation by ascorbate. To test this, PAT cells were grown for one week starting at a high cell density, 5×10^6 cells/flask (25 cm), and at the usual density of 1.2×10^6. The effect of initial cell density on the percentage of collagen synthesis is shown in Fig. 2 for medium F12 containing serum alone, serum plus lactate or serum plus ascorbate. In all cases, a high initial cell density helped to stabilize the percentage of collagen synthesis in the early part of the week. With ascorbate present, no drop in percent collagen synthesized

was observed. Thus high cell density by itself--or factors present at high density--are necessary for stability of collagen synthesis in these cells.

In summary, PAT cells in culture respond to ascorbate by increasing the percentage of collagen synthesis in a manner similar to cells *in vivo*. Slower growth rate does not necessarily lead to higher level of collagen synthesis. A high cell density is a necessary but not a sufficient condition to stabilize the differentiated state in these cells. A combination of high density and ascorbate stabilizes collagen synthesis at 25-30% for one week in culture.

Modulation of Collagen Synthesis

When PAT cells are placed in culture under conditions other than that described above, there is a drop in the percentage of collagen produced (5). This loss of function which occurs when all cells are placed in culture may or may not be reversible (3,4,16,20,21). In this paper, we refer to irreversible changes as terminal dedifferentiation and reversible events as modulations. Placing PAT cells in a detrimental medium which leads to synthesis of a low percentage of collagen and then switching them to a favorable medium should determine whether or not the synthesis of a high percentage of collagen is a reversible event. PAT cells were grown in F12 with either 0.5% or 3% serum but without ascorbate for four days and then half of the flasks were switched to the favorable conditions of F12, 0.5% serum plus ascorbate. As a further control, the reciprocal switch was also carried out: five days with ascorbate, two days without ascorbate in F12 plus 0.5% serum. The data presented in Fig. 3A and 3B show that collagen production is a modulated function in these cells. Altering the environment of the cell clearly affects the percentage of collagen produced within 24 hrs.

Moreover, even the detrimental effect of higher serum levels is fully reversible. It should be pointed out that the addition of ascorbate to medium containing 3% serum is not sufficient to induce a higher percentage of collagen (data not shown). In other words, the inhibitory effect of serum is more dominant than the positive effect of ascorbate.

As the cells reached a very high density there was a slight but significant decrease in the percentage of collagen produced (Fig. 3). This decline at the very end of the week appeared to be dependent on the final cell density in the flask which in turn was related to initial serum levels (and the particular batch of serum). Preliminary experiments indicate that this may be due to an inability of standard cell culture medium to nutritionally support cell function at this high cell density.

Ascorbate Affects the Percentage of Collagen Synthesized by Specifically Increasing the Absolute Level of Collagen Synthesis

We have so far expressed the synthesis of collagen as a percent of total protein. This is a critical value in determining whether PAT cells are in a normally differentiated state. In addition, the treatment of the data as a percentage corrects for fluctuations in the uptake of proline and overall metabolism. However, changes in percent value can be brought about by either altering the absolute level of collagen synthesized or total amount of protein synthesized. Either case has implications for the mode of regulation of collagen biosynthesis in PAT cells. In order to determine whether or not ascorbate increases the absolute level of collagen synthesis, it is important to consider any additional effects that it may induce in cultured cells. The presence of ascorbate in the medium did not seem to affect the overall cellular metabolism. This is reflected in the fact that growth rate was unaffected by the presence of ascorbate. In addition, ascorbate did not alter the rate of proline uptake: in the early part of

the week, the total incorporation of ^{3}H proline into protein (as well as the percentage of collagen produced) was comparable in control and ascorbate-containing cultures. Thus the rate of incorporation of ^{3}H proline into collagen and non-collagen proteins after ascorbate addition can be compared with minimal possibility of artifacts.

The absolute synthesis of collagen and non-collagen proteins was calculated from the data obtained in the experiment previously presented in Fig. 3A. In Table 1, two cases are presented: a comparison between cells on day 2 and day 4 which were grown in medium with and without ascorbate; and a comparison of cells which were or were not modulated with ascorbate for one day (day 4 to 5). The two situations chosen represent conditions where the cell changes the percentage of collagen synthesis most dramatically. The data shows that in both cases the addition of ascorbate has a significant effect only on the rate of collagen synthesis. Since the growth rates for cells with and without ascorbate is unchanged, reporting the results on a per cell basis would not alter the conclusion: ascorbate affects the percentage of collagen synthesis by increasing the absolute synthesis of collagen.

Maintenance of Morphological Characteristics

In the final analysis, what makes a cultured tendon cell 'normal' may prove much more complex than the ability of these cells to produce the correct amount of collagen. A normal tendon *in ovo* has other characteristics such as a specific metabolic pattern and a characteristic morphology (8). With regard to the latter we have examined the ability of tendon cells to line up between long rows of collagen fibers similar to cells *in vivo*. In a plastic flask in culture, no clear orientation of the cells exists. This may be due to the surface to which the cells are attached. We coated the

surface of the flask with collagen to more closely mimic the natural situation *in ovo*. The morphology of cells grown on a regular plastic flask and on a flask coated with salt precipatated collagen is shown in Fig. 4. PAT cells on the collagen surface are highly oriented while those on a plastic surface are randomly directed (when the percentage of collagen synthesis was optimal, growth on collagen did not significantly alter the rate of collagen synthesis (data not shown). This result clearly indicates that morphology as well as function can be manipulated in culture to more accurately correspond to tendon cells *in ovo*.

DISCUSSION

A differentiated cell must be defined in experimental terms so that it may be studied in culture. In the past, such a definition had confined itself to the ability of the cell to express a given function and not to the quantitative expression of that function (3,22). This definition, however, has its limitation, both *in vivo* and in culture. To begin with, it assumes that all differentiated functions are unique to one cell type. This clearly is not the case. Many cells in the body have the capacity to synthesize some collagen. However, it is only a few cell types, for example tendon or bone, which have the mission and thus the capacity for collagen to be their major cellular product. In these cells, aside from the type, the quantity of collagen synthesized best defines the cell, i.e., quantity becomes quality.

In culture, a definition of differentiation which relies on the ability to express and not on the level of expression is even more inadequate. In addition to earlier work by Green *et al*. (9), Langness and Udenfriend (23) recently used cloned non-fibroblast cell lines to show that cells which were not known for their collagen production *in vivo*, indeed make appreciable

amounts of collagen in culture when compared to fibroblast cell lines. Therefore, the mere qualitative synthesis of collagen is a poor criterion of differentiation for fibroblasts in tissue culture.

We define a differentiated cell in culture not only in terms of its ability to express tissue specific function, but also in terms of its potential to express function at a level and percentage comparable to that in vivo. Quantity of synthesis is not just an arbitrary definition of differentiation. This definition reflects the fact that for a cell to function normally in vivo, it must regulate presisely both the type and the quantity of its products. The percentage value is also critical because it accounts for the fact that in a tissue the synthesis of each component is regulated in amounts relative to other components. While numerical differences in levels of synthesis have generally been ignored as being insignificant, they may indeed signal important changes in the general state of the cell. For instance, cells synthesizing high levels of collagen respond to their environment differently than cells making lesser amounts. Thus, PAT cells and cells in vivo respond to ascorbate by increasing the synthesis of collagen; established cell lines, which make collagen at a much lower level, respond by merely increasing the activity of prolyl hydroxylase.

Maintenance of the differentiated state in a proliferating cell population outside the body was the original aim of cell culturists (4). While cells in general will either not grow or will lose their ability to express function, PAT cells are capable of both growing and maintaining their function if conditions are properly modified. However, the success in retaining full expression of function in PAT cells may reflect the fact that a tendon is a relatively simple tissue, made of only one kind of cells (fibroblasts) whose main function is to form collagen fibers. It is expected

0 0 0 0 4 8 0 2 9 4 3

that complex tissues such as liver, which have diverse functions and an intricate structure, will require a much more complex microenvironment for the maintenance of the differentiated state.

Despite the importance of achieving stable conditions for expression of function, the drop in function that occurs in primary cell cultures could be looked at as an asset rather than a liability: It contains information about the regulation of that function. A eukaryotic cell with a diminished or lost function can be looked at as the next best thing to a "mutant" providing a unique insight into important physiological questions. This is true, of course, only if the loss can be modulated. An irreversible loss cannot be used to study the regulation of function. In other words, both positive and negative modulations are needed. PAT cells are an ideal system in this respect. First, they produce collagen at a level which is more than an order of magnitude greater than commonly used cell lines: 30 versus 2% (5,11). Second, they can modulate the level of collagen synthesis from 5 to 30% depending on the cell density and the concentration of ascorbate, lactate, and serum. Third, they modulate not only the synthetic function, but also morphological orientation. And fourth, they respond to modulation in a manner similar to cells *in vivo*.

The mechanism by which PAT cells translate an environmental change to a change in the percentage of collagen synthesis is unclear at this point. A mechanism which relates a slow down in growth rate to a "turn on" of differentiated function would be unsatisfactory because a reciprocal relationship is not always observed. Any model for the regulation of collagen synthesis must be complex enough to account for the necessary involvement of both high density and ascorbate. To decipher the actual mechanism will require a more thorough understanding of the metabolic state of cells at high density

and the mechanism of action of ascorbate.

There are at least three further questions which may be raised with regard to this research. The first is how long could the cells remain fully responsive to changes in their environment. Could collagen synthesis be manipulated in the same fashion after second or third subcultures? Does "terminal dedifferentiation" ever need to occur? The second is whether other less dominant differentiated functions of tendon cells are controlled coordinately or separately when compared to collagen synthesis. The third, and much more complex question concerns the reversibility of development. If the environment of the cell is correctly modified, can a tendon cell undergo transdetermination (25) to become a totally different cell type? While these questions are not all directly related to the maintenance of a differentiated cell in culture, the answers are critical for an understanding of the mechanisms of gene regulation and the intriguing process of development.

0 0 J 0 4 8 0 2 9 4 4

ACKNOWLEDGEMENT

We would like to thank Deborah Farson for her excellent technical and intellectual assistance. This investigation was supported in part by the Division of Biomedical and Environmental Research of the U. S. Energy Research and Development Administration and in part by a National Institutes of Health Fellowship (R.I.S.), 1F32 CA05807-01, from the National Cancer Institute.

REFERENCES

1. Ursprung, H. (1968) The Stability of the Differentiated State, (Springer-Verlag, New York).
2. Eagle, H., (1965) "Metabolic Controls in Cultured Mammalian Cells," Science 148, 42-51.
3. Green, H. and Todaro, G.J., (1967) "The Mammalian Cells as Differentiated Microorganism," Ann Rev. of Microbiol. 21, 573-600.
4. Davidson, E.H., (1964) "Differentiation in Monolayer Tissue Culture Cells," Advanc. Genet. 12, 143-280.
5. Schwarz, R., Colarusso, L., and Doty, P., (1976) "Maintenance of Differentiation in Primary Cultures of Avian Tendon Cells," Expt. Cell. Res. 102, 63-71.
6. Schwarz, R., (1975) Ph.D. Thesis, Harvard University.
7. Dehm, P., and Prockop, D.J., (1971) "Synthesis and Extrusion of Collagen by Freshly Isolated Cells from Chick Embryo Tendon," Biochim. Biophys. Acta 240, 358-369.
8. Bloom, W. and Fawcett, D.W., (1975) A Textbook of Histology (W.B. Saunders, Philadelphia) 10th ed..
9. Green, H. Goldberg, B., and Todaro, G.J., (1966) "Differentiated Cell Types and the Regulation of Collagen Synthesis," Nature 212, 631-633.
10. Green, H., and Goldberg, B., (1964) "Collagen and Cell Protein Synthesis by an Established Mammalian Fibroblast Line," Nature 204, 347-349.
11. Peterkofsky, B., (1972) "The Effect of Ascorbic Acid on Collagen Polypeptide Synthesis and Proline Hydroxylation During the Growth of Cultured Fibroblasts," Arch. Biochem. Biophys. 152, 318-328.
12. Peterkofsky, B., and Diegelmann, R., (1971) "Use of a Mixture of Proteinase-Free Collagenases for the Specific Assay of Radioactive Collagen in the Presence of Other Proteins," Biochemistry 10, 988-994.

0 0 0 0 4 8 0 2 9 4 5

13. Green, H. and Goldberg, B., (1965) "Sythnesis of Collagen by Mammalian Cell Lines of Fibroblastic and Nonfibroblastic Origin," Proc. Nat. Acad. Sci. USA 53, 1360-1365.

14. Levine, C.I., and Bates, C.J., (1975) "Ascorbic Acid and Collagen Synthesis in Cultured Fibroblasts," Ann. N.Y. Acad. Sci. 258, 288-306.

15. Evans, C.A., and Peterkofsky, B., (1976) "Ascorbate-Independent Proline Hydroxylation Resulting from Viral Transformation of Balb 3T3 Cells and Unaffected by Dibutyryl cAMP Treatment," J. Cell. Physiol. 89, 355-368.

16. Peterkofsky, B., and Prather, W.B., (1974) "Increased Collagen Synthesis in Kirsten Sarcoma Virus-Transformed Balb 3T3 Cells Grown in the Presence of Dibutyryl Cyclic AMP, Cell 3, 291-299.

17. Ham, R.G., (1965) "Clonal Growth of Mammalian Cells in Chemically Defined, Sythetic Medium," Proc. Nat. Acad. Sci. USA 53, 288-293.

18. Barnes, M.J., (1975) "Function of Ascorbic Acid in Collagen Metabolism," Ann. N.Y. Acad. Sci. 258, 264-277.

19. Bissell, M.J., Hatie, C., and Rubin, H., (1972) "Patterns of Glucose Metabolism in Normal and Virus-Transformed Chick Cells in Tissue Culture," J. Natl. Cancer Inst. 49, 555-565.

20. Peterkofsky, B., (1972) "Regulation of Collagen Secretion by Ascorbic Acid in 3T3 and Chick Embryo Fibroblasts," Biochem. Biophys. Res. Commun. 49, 1343-1350.

21. Diegelmann, R.F., and Peterkofsky, B., (1972) "Collagen Biosynthesis During Connective Tissue Development in Chick Embryo," Dev. Biol. 28, 443-453.

22. Holtzer, H., and Abbott, J., (1968) "Oscillations of the Chondrogenic Phenotype in vitro," in The Stability of the Differentiated State, ed. Ursprung, H. (Springer-Verlag, N.Y.). 1-16.

23. Langness, V., and Undenfriend, S., (1974) "Collagen Biosynthesis in Nonfibroblastic Cell Lines," Proc. Nat. Acad. Sci. USA 71, 50.

24. Stassen, F.L.H., Cardinale, G.J., and Undenfriend, S., (1973) "Activation of Prolyl Hydroxylase in L-929 Fibroblasts by Ascorbic Acid," Proc. Nat. Acad. Sci. USA 70, 1090-1093.

25. Gehring, W., (1968) "The Stability of the Determined state in Cultures of Imaginal Disks in Drosophila" in The Stability of the Differentiated State, ed. Ursprung, H. (Springer-Verlag, N.Y.). 136-154.

FIGURE LEGENDS

Figure 1: Collagen synthesis of PAT cells over a one-week period in medium F12, 0.5% serum (▲) containing various additional factors: 50 ug/ml sodium ascorbate (●); 37 mM sodium lactate (○); 50 ug/ml sodium ascorbate and 37 mM sodium lactate (△); The ordinate (left) shows the percentage of incorporated ^{3}H proline which is sensitive to collagenase; (right) this value corrected for the 5.2 ratio of proline content in collagen to the other cellular proteins. The correction is based on the method used by Diegelmann and Peterkofsky (21).

Bottom: Growth curves for PAT cells in the above media.

Figure 2: Effect of initial cell density on the collagen synthesis of PAT cells. Flasks were innoculated with 5×10^{6} (●) and 1.2×10^{6} (O) cells and grown in F12 medium containing: A) 0.5% serum; B) 0.1% serum + 37 mM sodium lactate; C) 0.5% serum + 50 ug/ml sodium ascorbate. (The use of 0.1% instead of 0.5% serum in "B" is not critical; with lactate and certain batches of serum, the collagen level at the end of the week was slightly improved.)

Figure 3: Modulation of collagen synthesis by altering the medium. A) Two experiments are presented. Cells growing in medium F12, 0.5% serum + 50 ug/ml sodium ascorbate (●) were switched on day 5 to medium without ascorbate (O). Cells growing in F12, 0.5% serum (▲) were switched on day 4 to medium which also contained 50 ug/ml sodium ascorbate (△).

B) Cells growing in F12, 3% serum (▲), were switched on day 4 to medium F12 containing 0.5% serum + 50 ug/ml sodium ascorbate (△). (The initial low percentage of collagen synthesis is due to the presence of 3% serum (5).

Figure 4: Phase contrast micrographs of PAT cells on day 3, grown in F12, 0.5% serum, + 50 ug/ml sodium ascorbate. A) Cells attached to a plastic flask coated with collagen. B) Cells attached to a regular plastic flask. It should be pointed out that the orientation of the cells does not extend continuously across the flask but instead occurs in many patches. This limitation we relate to our preliminary method of precipating and orienting the collagen coating on the flask.

Footnote to Table 1: The data used in preparing Figure 3a has been recalculated to present absolute incorporation into collagen and non-collagen proteins. The data is presented as "corrected CPM", which adjusts for the use of radioactive proline as the probe (see legend, Figure 1).

0 0 0 0 4 8 0 2 9 4 6

TABLE 1

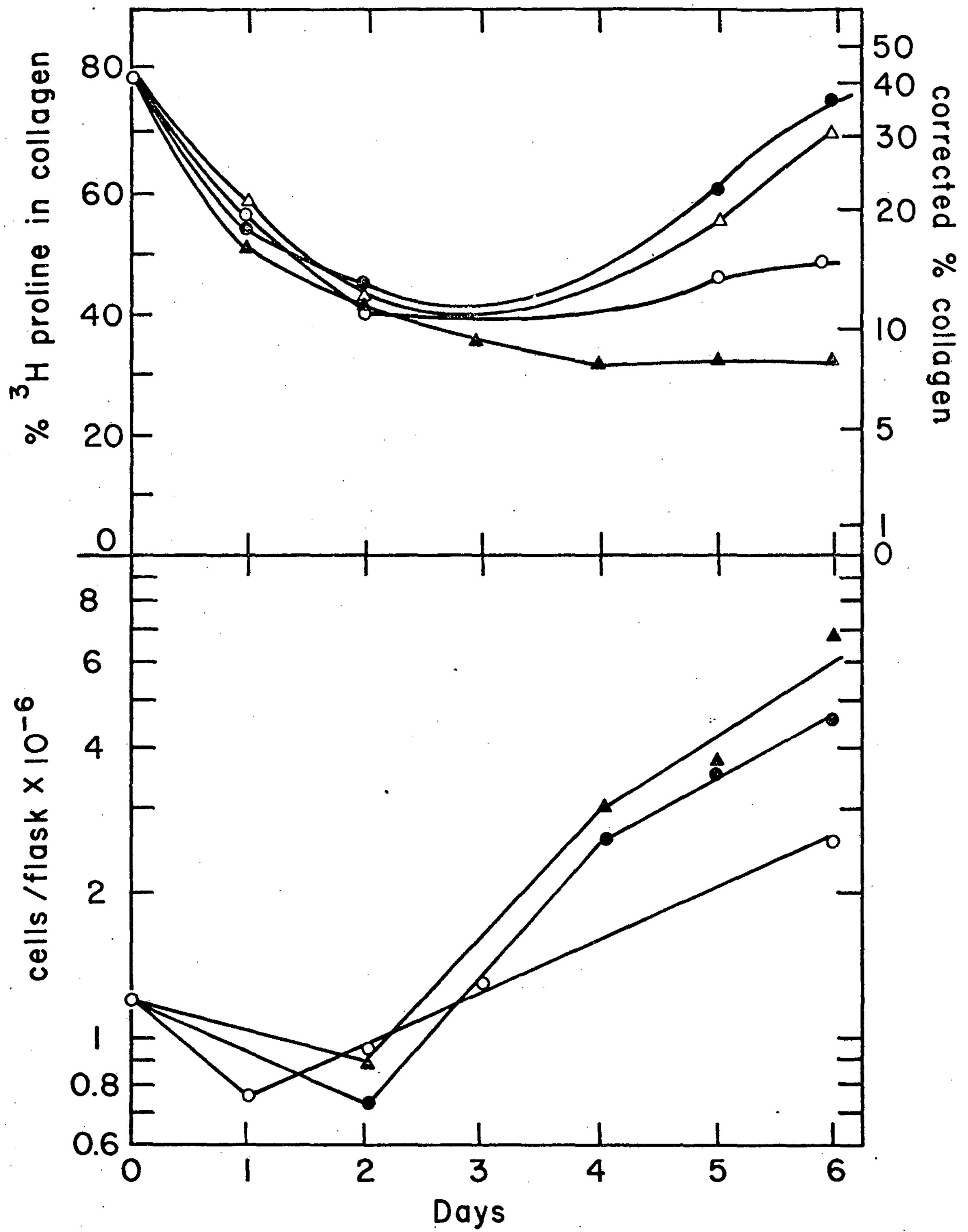
EFFECT OF ASCORBATE ON ABSOLUTE COLLAGEN AND NON-COLLAGEN PROTEIN SYNTHESIS

A. Cells grown with and without ascorbate (c)

	day 2 corrected CPM incorporated/flask		day 4 corrected CPM incorporated/flask		ratio (day 4/day 2)	
	-c	+c	-c	+c	-c	+c
collagen	4500	8380	8110	43060	1.8	5.1
non-collagen protein	54830	68580	73360	92740	1.3	1.3
corrected % collagen	8	11	10	32		

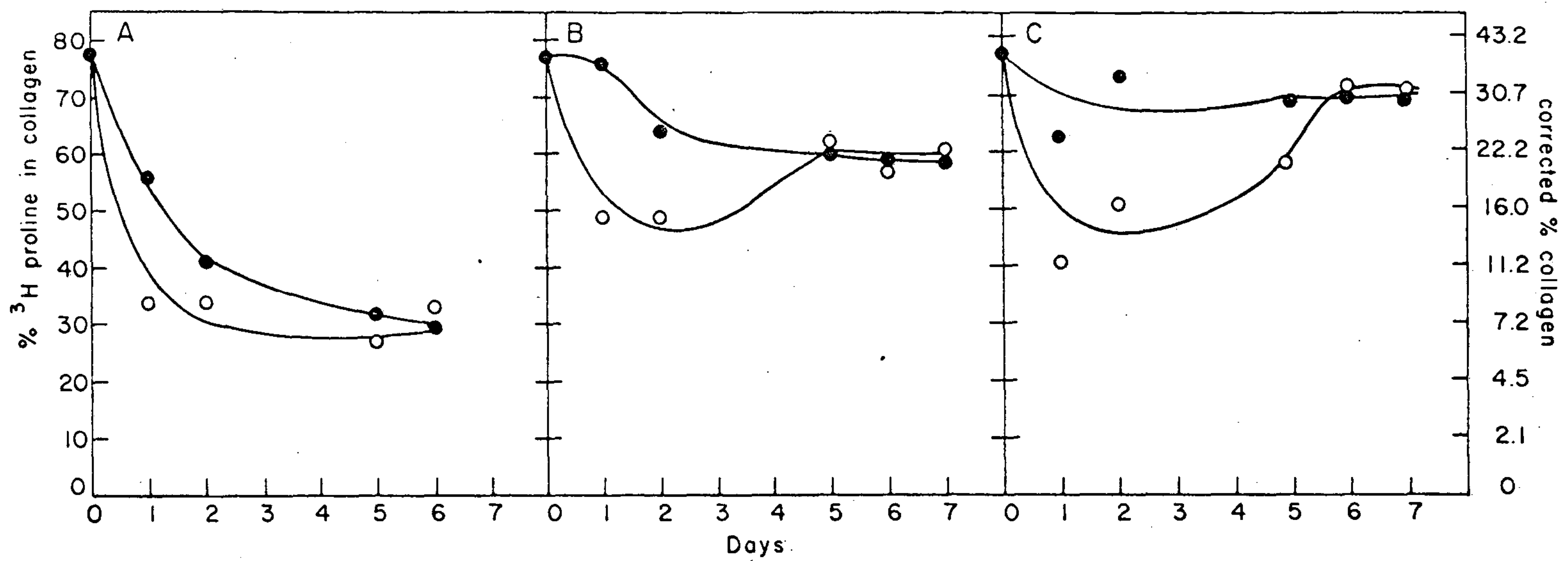
B. Cells modulated with ascorbate on day 4

	day 4 corrected CPM incorporated/flask		day 5 corrected CPM incorporated/flask		ratio (day 5/day 4)	
	control	modulated	control	modulated	control	modulated
collagen	8110	12790	18570	47870	2.3	3.7
non-collagen proteins	73360	106720	165440	180650	2.3	1.7
corrected % collagen	10	11	10	21		



Schwarz &
Bissell

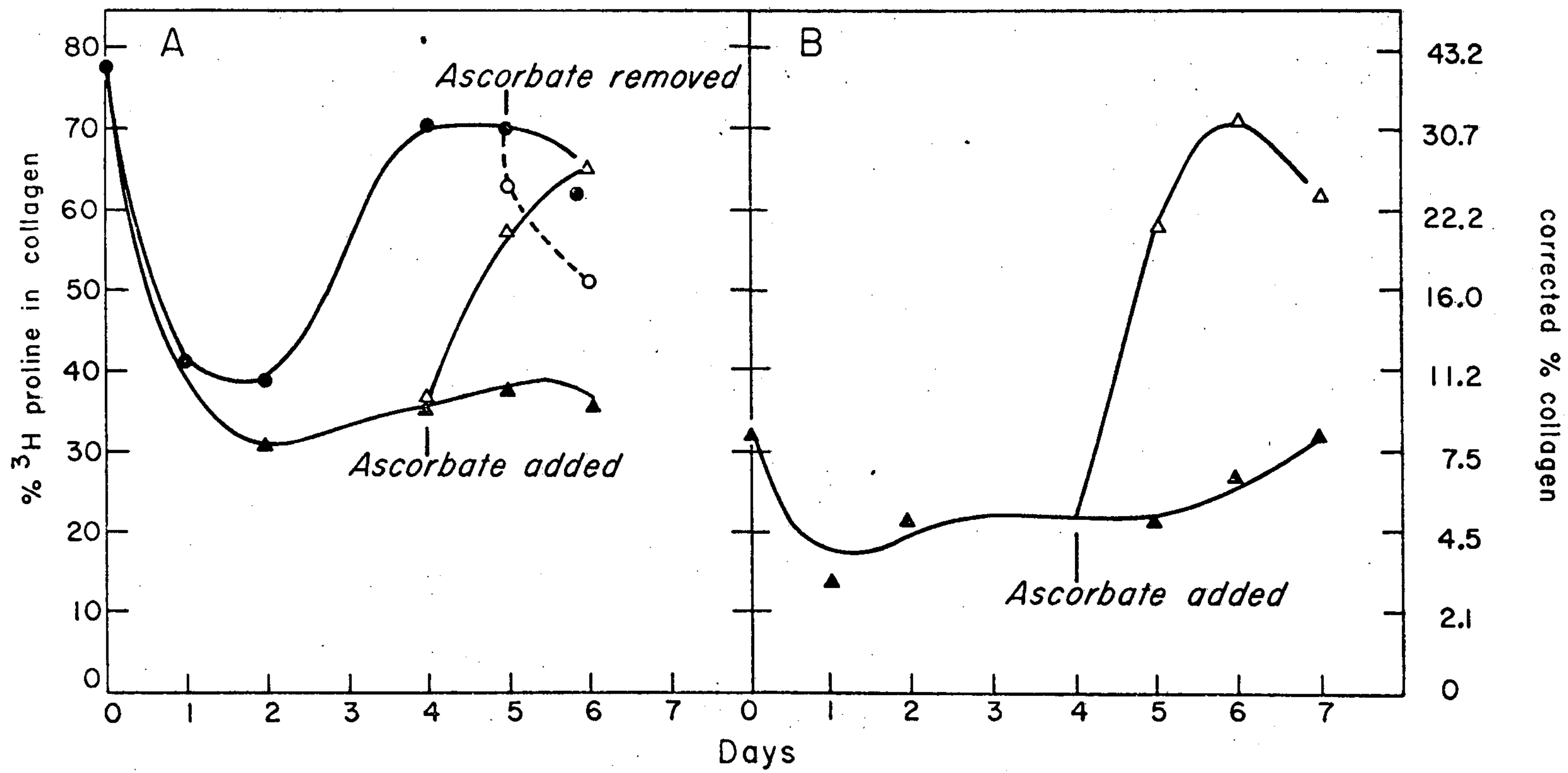
Figure 1



Schwarz &
Bissell

Figure 2

0 0 0 0 4 8 0 2 9 4 7

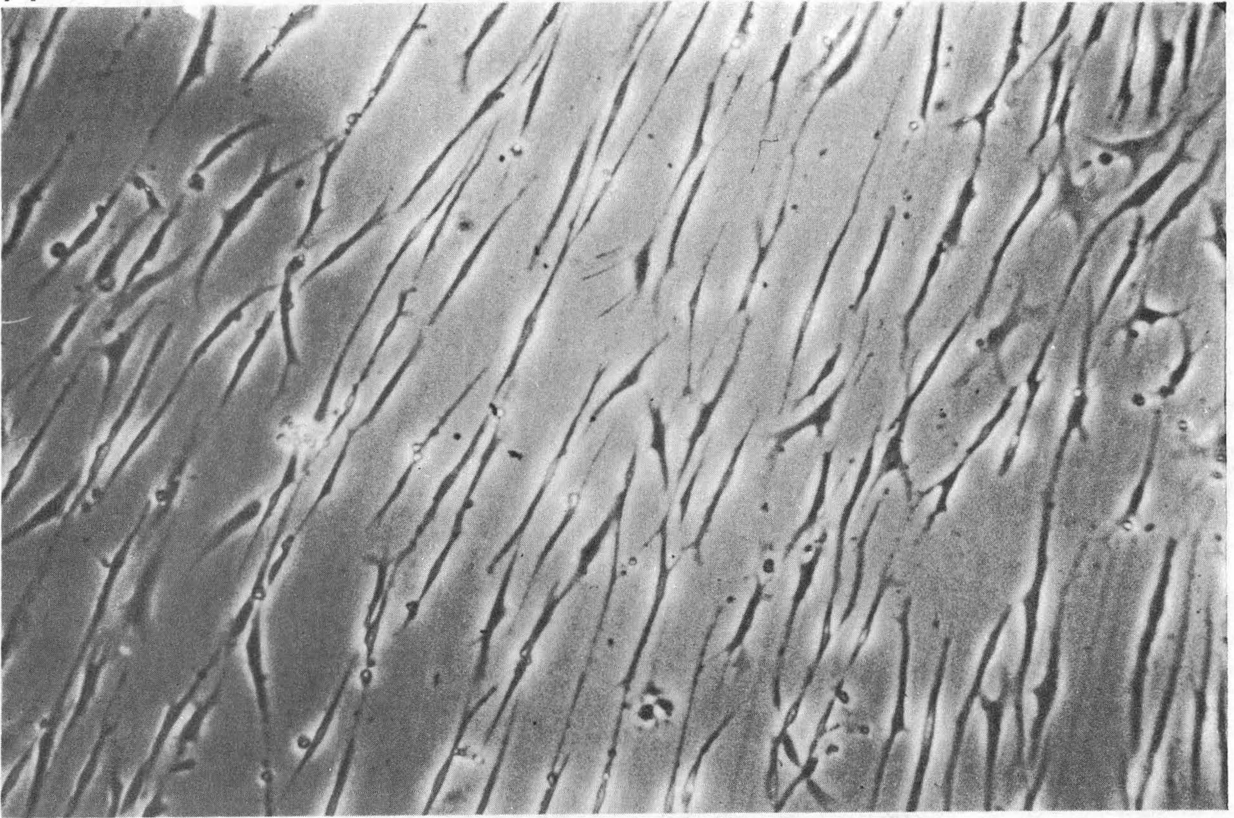


Schwarz &
Bissell

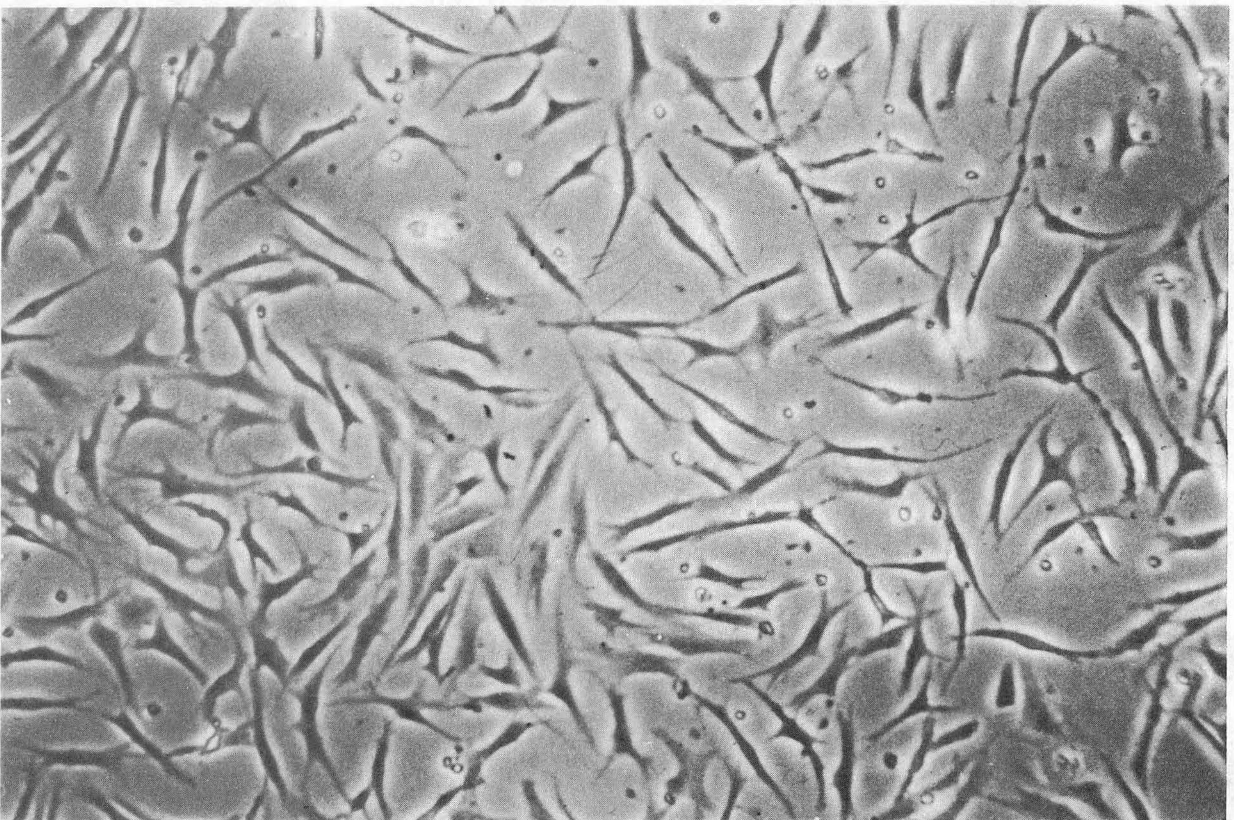
Figure 3

0 0 0 0 4 8 0 2 9 4 8

A



B



100μ

XBB 771-259A

Fig. 4

U U U U 4 8 U 2 7 4 9

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720