Lawrence Berkeley National Laboratory
Recent Work

Title
REQUIREMENTS FOR MAINTAINING THE EMBRYONIC STATE OF PAT CELLS IN CULTURE: A NEW MODEL FOR AGEING AT THE CELLULAR LEVEL

Permalink
https://escholarship.org/uc/item/2t3730b0

Author
Schwarz, Richard I.

Publication Date
1978-08-01

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

UC-48

Submitted to DEVELOPMENTAL BIOLOGY

LBL-8165 c.1

REQUIREMENTS FOR MAINTAINING THE EMBRYONIC STATE OF PAT CELLS IN CULTURE: A NEW MODEL FOR AGEING AT THE CELLULAR LEVEL

Richard I. Schwarz, Deborah A. Farson, and Mina J. Bissell

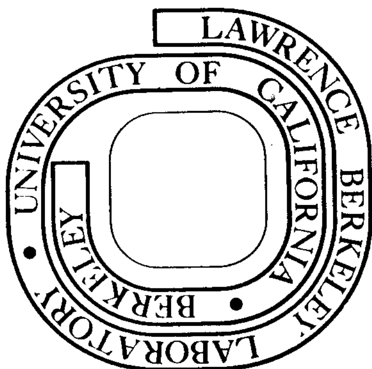
August 1978

Prepared for the U. S. Department of Energy under Contract W-7405-ENG-48

For Reference

Not to be taken from this room

RECEIVED
LAWRENCE
BERKELEY LABORATORY
JAN 29 1979
LIBRARY AND
DOCUMENTS SECTION



LBL-8165 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 0 0 5 1 0 7 7 9 4

REQUIREMENTS FOR MAINTAINING THE EMBRYONIC STATE OF PAT CELLS IN CULTURE: A NEW MODEL FOR AGEING AT THE CELLULAR LEVEL

Richard I. Schwarz, Deborah A. Farson, and Mina J. Bissell

Laboratory of Chemical Biodyanmics

Lawrence Berkeley Laboratory

University of California

0 0 0 0 5 1 0 7 7 9 5

ABSTRACT

Primary Avian Tendon cells (PAT) will maintain their embryonic state on being cultured under correct conditions; that is, they retain the potential for devoting 20-30% of their total protein synthesis to collagen. However, if the cells remain at confluency or are derived from confluent cultures, this potential is irreversibly decreased. This effect, along with poor medium formulations, probably accounts for the "dedifferentiation" process which occurs when fibroblasts are cultured.

This loss of function has long been considered to be a cell culture artifact; however, we consider that this drop in collagen synthesis is a reflection of a developmental phenomenon. We show that embryonic tendon cells *in ovo* make over 30% collagen but adult tendon cells make less than 1%. This drop is an essential phenomenon in an animal with determinate growth potential, for without it, the tendon would continue to enlarge beyond a useful size. Cultures of embryonic tendon cells appear to be triggered to "age" by a mechanism which correlates with high cell density.

Moreover, the low levels of differentiated synthesis in mature tendon cells would severely hamper the ability of these cells to renew damage. We postulate that similar effects, in many different tissues, would account for senescence.

INTRODUCTION

When placed in culture, fibroblasts lose their ability to synthesize collagen (Peterkofsky, 1972b, Schwarz et al., 1976). With time, the level of collagen drops and approaches a residual collagen level of 1% of the total protein synthesized (Peterkofsky, 1972a). While this loss of differentiated function in cultured cells has been observed for over 70 years, and in many cell types, the underlying cause for this decline has remained obscure (Davidson, 1964). A solution to this problem would yield a cell system where analysis of the regulation of differentiated function is significantly simplified: a stabilized function synthesized in abundance.

In previous papers (Schwarz et al., 1976; Schwarz and Bissell, 1977), we have shown that for PAT cells (primary avian tendon, from 16-day old chick embryos), at least a partial explanation for a loss of normal function was a deleterious selection of the medium based solely on the requirements for growth. Medium additionally selected for the expression of differentiated function (low serum levels and the inclusion of ascorbate) allowed PAT cells to continue to synthesize 20-30% of their protein synthesis as collagen, a situation which approximates the in ovo state.

However, while these results pointed to a need for more careful selection of the growth medium, a change in medium alone could not completely explain the decline in differentiated function in cultured cells. Because in primary cultures, we found that cells first grown in an unsatisfactory medium were able to synthesize high levels of collagen when the medium was corrected (Schwarz and Bissell, 1977). We were led, therefore, to explore longer term cultures of PAT cells in order to understand the cause of the irreversible low levels of collagen synthesis seen in most fibroblast cell lines.

0 0 0 0 5 1 0 7 7 9 6

In this paper, we show that confluent cells or cells derived from confluent cultures have different synthetic abilities than the original primary culture or subcultures derived from low density cultures. Cells maintained at a high cell density irreversibly lose their potential for synthesizing high levels of collagen. This effect probably accounts for a major part of the "dedifferentiation" which occurs when cells are cultured.

While the loss of function on culturing cells has been considered to be in a large part an artifact, we relate this cellular response - a loss in ability to synthesize high levels of collagen - to part of the inherent developmental programming of normal tendon cells. This is for two reasons: one, in previous research (Schwarz and Bissell, 1977) we have modified the medium such that it is adequate for fully maintaining differentiated protein synthesis in these cells; two, we show in this paper that as tendon cells *in vivo* mature from an embryonic to an adult stage, their ability to synthesize collagen is decreased. Cultured embryonic tendon cells at confluency, therefore, appear to be similarly triggered to age, with respect to their synthetic pattern. We conclude that the ability of a tendon cell to regulate the percentage of collagen produced has important implications both for the process of development and for the occurrence of senescence.

MATERIALS AND METHODS

Isolation of Cells and Cell Culture: Tendon cells were isolated from 16-day chick embryos as previously described (Dehm and Prockop, 1972; Schwarz *et al.*, 1976). Cells were grown in 25 cm^2 flasks in F12 medium (Ham, 1965) supplemented with fetal calf serum and ascorbic acid (50 μg/ml). The initial innoculum of cells and the level of serum is detailed in the figure legends. Other details have been described previously (Schwarz and Bissell, 1977).

Subculturing and Cell Counting: Cells were detached from the surface of the flasks using a 1:1 ratio of the same enzyme mixture (collagenase 0.3%, and trypsin 0.15%, in medium F10 plus 5% fetal calf serum) used to initially isolate the cells from the intact tendon (Schwarz *et al.*, 1976), and a pancreatin (2.5%, Gibco) solution. In order to remove the cells, flasks were incubated in 1 ml at 38°C for times which varied from 15 min up to 1 hr. The longer times were without any apparent detrimental effects on the plating efficiency which approached 100%. After most of the cells had detached, the cells were gently pipetted to dissociate clumps and to remove any remaining cells. Cells were pooled, centrifuged twice, and resuspended each time in F12 medium. After this step, the cells were treated identically to primary cultures.

A similar procedure was used for removing cells for counting on a Coulter Counter. However, longer incubation times and more vigorous pippetting were used to further reduce clumping.

Collagen Assay: For cultured cells, flasks were pulsed with 1 ml of fresh medium containing 50 μCi of ^{3}H-proline (2'-3', New England Nuclear) for 3 hr. Collagen was assayed by a collagenase method (Peterkofsky and Diegelmann, 1971) modified as previously described (Schwarz, *et al.*, 1976). 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

0 0 0 0 5 1 0 7 7 9 7

for every experiment; instead, a mean (± S.D.) point derived from previous experiments (9) was used (75 ± 6).

For intact tendons, collagen was assayed by pulsing with 10 μCi of ^{14}C-proline (New England Nuclear) in 2 mls of F12 medium for 3 hr. Then the tendons and medium were dialyzed exhaustively against distilled water. The dialyzed tendons were dehydrated under vacuum and heat (50°C). Two ml of constant boiling HCl (Pierce) was added and the tube was sealed under vacuum. The protein was hydrolyzed for 24 hr at 105°C. Approximately 5,000 CPM were spotted on a paper chromatogram and were run in two dimensions as described previously (Bassham *et al.*, 1974). The proline and hydroxyproline spots were widely separated, were cut out, eluted with water and counted in aquasol 2 (New England Nuclear) in a liquid scintilation counter.

Eggs and Chickens: Fertilized eggs were obtained from Western Scientific Supply. A young fryer chicken (under 6 months old) was obtained from the On Sang Poultry Co. (San Francisco, CA). Two old stewing chickens (4 to 5 years old) were obtained from Mr. and Mrs. Young of El Sobrante, CA.

RESULTS

Collagen Synthesis in Primary Cultures: When PAT cells are placed in primary culture at low innoculum and grown in medium optimal for collagen synthesis, one obtains an extremely reproducible curve for the level of collagen synthesis versus time (Schwarz and Bissell, 1977). Four distinct periods are observed and this is shown in Fig. 1. The first, labeled "A", is concerned with cells when they are first placed in culture. These cells are unique in that independent of cell density they will devote a large percentage of their protein synthesis to collagen (35%). During other periods, a high cell density is essential for a high level of collagen synthesis (Schwarz and Bissell, 1977). PAT cells appear to retain for less than one day the information that they were derived from an *in ovo*, high cell density situation. For by the next day, the level of collagen synthesis drops, reflecting the fact that the cells were seeded at a low cell density. We have shown previously that in the presence of ascorbate this drop only occurs when cells are at low density (Schwarz and Bissell, 1977). This time of low density and low collagen synthesis is the "B" period. As the cells grow and approach confluency, the level of collagen synthesis increases and this we refer to as the "C" period. Once confluent, the length of time cells will continue to make *in ovo* levels of collagen, is variable. The length of this period depends on several factors but has ranged from 0 to 7 days. This will be discussed in more detail below. PAT cells, in any case, will not remain making high levels of collagen at a high cell density. The level eventually falls from 25-30% to about 7-15% and this is the final "D" period.

To see whether or not the decline in the "D" period is the result of a failure of the medium to support cells at a high density, we doubled the medium

volume in an attempt to alleviate any nutrient deficiency (the medium was already being changed daily). This additional change had no effect (data not shown). The inability of PAT cells to sustain a high level of collagen synthesis, therefore, could not easily be related to a nutrient deficiency,

For most experiments involving short-term primary cultures, the "D" period could be avoided by plating cells at lower initial densities, thereby extending the period ("B") of low density and rapid growth. However, it became apparent that with different batches of serum at the same concentration, the length of the "C" period (of high collagen synthesis) could vary immensely and in certain cases even disappear all together. A test of each batch of serum for an optimal concentration, which would sustain a sufficient period of high collagen synthesis, became necessary. An example of this type of test is shown in Fig. 2. In this case, three serum concentrations were used: 1%, 0.5%, and 0.25%, and collagen assays were only performed at the end of the week (day 5 through 8), in order to gauge the extent of the "C" period. The results show that the higher serum levels have dramatic negative effects on both the percentage of collagen synthesis and on the length of the "C" period. While with the batch of serum used in this experiment (Fig. 2) lowering the level of serum appears to be increasingly effective in maintaining a high level of collagen synthesis, the reduction in serum concentration has its limitations. For instance, PAT cells without serum will multiply slowly (Schwarz et al., 1976), but will not produce collagen at levels greater than 15% when grown to high cell density (data not shown). For most batches of serum, the optimum concentration is between 0.1% and 0.5%.

The behavior of cells in the "C" period seems to be governed by three parameters: cells need a reasonable metabolic rate, a high degree of density dependent inhibition, and synchrony of growth. The need for a reasonable metabolic rate, we relate to the fact that very low or no serum medium is ineffective in stimulating PAT cells in the "C" period to make a

high percentage of collagen. Serum levels below optimum affect the metabolic rate as reflected by a lowered growth rate and a lowered level of protein synthesis (Schwarz et al., 1976). The dependence on strict monolayer cultures correlates well with the changes that occur when serum levels are raised above the optimum. Excess serum, while not affecting the exponential growth rate in the early part of the week (Schwarz et al., 1976), dramatically increases the final density reached at the end of the week. Why a tendency to overgrow a monolayer should facilitate the transition from the "C" stage to the "D" stage is unknown. The requirement for a synchronous growth pattern stems from the fact that 30% collagen synthesis appears to be close to the maximum that PAT cells can produce in culture (and possibly *in vivo*). For a flask of cells as a whole to make 30% collagen requires that all the cells in the flask makes 30% collagen. Thus, if the cells are plated unevenly, and reach high density at different times, the effect will be to shorten the "C" period.

Secondary Cultures: In the previous section, one observes that maintaining long-term primary cultures will not result in maintenance of function. This is a severe disadvantage in those experiments which require longer term cultures. We, therefore, became interested in whether subculturing of confluent monolayer would extend the culturing period and still maintain the level of differentiation observed in primary cultures (Fig. 1). Moreover, we were interested in determining whether or not the loss of function observed in the "D" period was reversible. If the cells could recover on being subcultured, then this would suggest that the loss of function was an artifact of culture conditions.

Before making secondary cultures, we needed a method for detaching the cells from the flask without causing permanent functional harm. Since cells could be isolated from a tendon without apparent injury using a

collagenase and trypsin mixture in F10 with 5% serum, we used the same procedure on tendon cells in culture. Under these conditions some cells would come off from the bottom of the flask but most remained bound. Trypsin (.05%) in a Ca^{++} free, buffered salt solution was reasonably successful, but the time needed for cell removal was very close to the point where cell lysis could occur. Finally, we found that a mixture of collagenase, trypsin, and pancreatin in F10 with 2.5% serum (see Methods) worked well in detaching the cells while retaining viability.

PAT cells were then removed from flasks of confluent monolayer (in the "C" period) which had been in culture for seven days. These cells were returned to new flasks at lower density and allowed to grow--a typical situation for secondary cultures. These cultures behaved very similarly to primary cultures with the following exceptions. They had a shorter lag time before exponential growth began; they did not have a spindled morphology on first attaching to the flask as did primary cells; and, most importantly, the level of collagen sythesis while showing the same dip and raise which was so characteristic of the cell density dependence of collagen synthesis, did not go above 15% in confluent cultures.

Two simple possibilities existed which might be the cause of the inhibition which stopped secondary cultures from producing collagen to the same level as primary cultures. The first was that the solution used to detach the cells caused irreversible harm. The second thought was that the serum requirements had changed. Investigations which should have mitigated these problems proved unsuccessful; therefore, other possibilities were pursued.

One approach, stemming from the beneficial effect that splitting subconfluent cultures had on the mouse 3T3 system (Todaro and Green, 1963), was that using cells at subconfluency might improve the situation. To test this possibility, PAT cell cultures were split on day 4 and day 7,

using identical treatments and replated at a low cell density of 0.6×10^6 cells per flask. The data from this experiment are shown in Fig. 3. One observes that splitting a subconfluent population yields cells which behave almost identically to primary cells and achieve a level of collagen synthesis which is close to (and in this particular experiment, better than) the primary culture. When confluent cultures were subcultured, the cells obtained reached a maximum level of collagen synthesis of only 15%.

One can conclude two important points from the above experiment. One, if PAT cells are to retain the synthetic capacity for collagen synthesis that they had in the embryo, then subculturing must be restricted to subconfluent cultures. Second, the change that occurs at confluency which results in a lowering of the level of collagen synthesis appears to be a genetic or epigenetic change. This is reflected by the fact that the change is transmittable to succeeding generations and that it occurs in a permissive environment - a medium which allows the full expression of differentiated function in PAT cells.

Tertiary Cultures: Having determined that one cause for a decline in differentiated function was due to growing cells to confluency before subculturing, we decided to expand the experiment to tertiary cultures to determine if any other variables were important. Two factors which were not clearly resolved in the previous experiment (Fig. 3), were whether or not time in culture or number of cell divisions would become limitations in the ability of PAT cells to express collagen.

To test these factors experimentally meant growing cells from very low densities. In this way, cells would require more time and would achieve more cell divisions before reaching a critical subconfluent density, at which point they would have to be transferred in order to maintain function. However, PAT cells in F12 medium at low serum would not grow if seeded

0 0 0 0 5 1 0 7 8 0 0

below 2×10^5 cells/flask without ascorbate, and 1×10^5 if seeded with ascorbate (this effect of ascorbate on tendon cells was opposite to that observed for chick embryo fibroblasts [Peterkofsky and Prather, 1977]). Within this limitation, PAT cells were grown in F12 medium supplemented with 0.2% fetal calf serum and 50 μg/ml ascorbic acid. As primary cells, where there was usually only a 50% plating efficiency, cells were seeded at 6×10^5 cells/flask. Since in secondary and tertiary cultures plating efficiency approached 100%; the density was reduced to 2×10^5. Cells were subcultured when they reached a density between 7×10^5 and 1×10^6 cells/flask. The data for levels of collagen synthesis and growth curves are shown in Fig. 4.

If we restrict ourselves first to the primary and secondary cultures, we observe a strong similarity in collagen synthesis and in growth curves. As discussed previously, one difference in secondary PAT cells is that they have a very short lag period before exponential growth. Therefore, the cells spend less time at low cell density and the drop in collagen synthesis due to a low cell density appears less intense. Nevertheless, in both primary and secondary cultures, high levels of collagen synthesis are obtained, about 25%. This experiment also shows that number of generations achieved in secondary culture can almost be doubled without affecting the ability of these cells to make collagen. Moreover, the length of time in culture has been increased by 50% with no significant effect. In addition, it should be pointed out that in both primary cultures on day 4 and secondary cultures on day 8, PAT cells can grow with a 24 hr generation time and make a fairly high percentage of collagen: approximately 18%. Thus, expression of differentiated function at this level and growth need not be incompatible.

When one looks at the tertiary cultures the pattern of collagen synthesis over a 7-day period appears much more complicated because the tertiary

cultures never make more than 16% collagen. However, this result need not be looked upon as an indication of the inherent negative effects of cell culture, but instead is probably due to the inability of serum at these low concentrations to remain a potent mitagen. As discussed previously, a fairly rapid metabolic rate is necessary for PAT cells to recognize a high density situation and synthesize a high level of collagen. One way to judge this stimulatory effect of serum is to observe at what cell density PAT cells will maintain a 24 hr generation time before becoming density inhibited.

As shown in Fig. 4B, primary cultures will grow to 1.4×10^6 cells/flask before slowing down the rate of cell division; as secondary cultures, this drops to 0.9×10^6 cells/flask; as tertiary cultures this drops to 0.4×10^6 cells/flask (and the most rapid growth rate never reaches a 24 hr generation time). With other batches of serum, this adaption to and negation of the stimulatory effect of serum has been seen to occur in secondary cultures, leading to lower levels of collagen synthesis (data not shown). This phenomenon, per se, does not relate to number of subculturings or time but only to the batch of serum. The fact that higher levels of serum can be detrimental to collagen synthesis (Fig. 2) leads to an experimental dilemma where an optimum concentration for tertiary cultures may not exist. The solution of this problem may only come from finding a more defined and consistent stimulator of the cells or selection of an uncommon lot of serum with better characteristics.

While a permanent maintenance of the embryonic differentiated state of PAT cells in culture has not been achieved, the important parameters have been been elucidated. These are the medium composition, subculturing prior to confluency and maintaining a rapid growth potential. At this time cultures of almost 2 weeks duration, which have gone over 4 generations, can be fully maintained so that they have the potential of producing 20-30% collagen.

0 0 0 0 5 1 0 7 8 0 1

Developmental Programming of Collagen Synthesis : The fact that PAT cells at high density have a tendency to change states, resulting in a reduction in the percentage of collagen produced, can be looked upon in two ways. One is that it is an artifact of cell culture. The other possibility is that this type of behavior is part of the developmental programming of the cell. In the rest of this section, we will present evidence for this latter possibility.

To relate a drop in collagen synthesis in culture to development, requires a theory which relates the production of collagen to the needs of the organism. Our premise is that an animal, such as a chicken, which has strict requirements for size (determinate growth) would express this on the cellular level by a restriction in the synthesis of structural proteins at the adult stage.

To test this prediction we isolated tendons from two 16-day embryos, a chicken under 6 months old, and two adult chickens approximately 5 years old. The tendons were pulsed with ^{14}C proline and the ratio of incorporated radioactive proline to hydroxyproline (a modified amino acid exclusively found in collagen in tendon tissue) was measured and used to calculate the percentage of collagen synthesis. The data is presented in Table 1. The level of synthesis for the 16-day embryo was in the same high range (25-35%) as that observed by Dehm and Prockop (1971) using 17-day embryos and a similar method. By about 6 months this had fallen by 1/2; and by 5 years, when full growth had long been achieved, the level was under 1%.

These results confirm the prediction that one important developmental step (in animals with determinate growth) is the reduction in the synthesis of structural proteins. Thus, the decline in collagen synthesis observed for tendon cells in culture at high density (the "D" period, Fig. 1) may be related to that observed for tendon cells *in vivo* with increasing age (Table 1).

DISCUSSION

Cells were originally put into culture as an approach to simplify the analysis of their in vivo behavior. Most cells, however, respond quickly and quite negatively to this radical change in their environment. Recently, it has become apparent that upon long-term culturing, cells change to such a degree that their relationship to their in vivo state is difficult to establish. These negative findings have led to skepticism over the use of cell culture as a general model for the in vivo state.

The basic problem stems from defining cell culture conditions almost exclusively for growth parameters. When PAT cells are analyzed by their ability to express differentiated function, one finds that these cells display a complex behavior independent of their ability to divide. PAT cells respond in a variety of ways to small changes in the composition of the medium, to subtle variations in serum levels, to the density of cells, and to the regime used in subculturing.

Being aware of these variables and by holding the critical ones constant, the results obtained in PAT culture do seem to accurately reflect the in vivo state. For example, in previous papers, we have shown that PAT cells respond negatively to the removal of ascorbate from the medium (Schwarz and Bissell, 1977). They decrease both the level of hydroxylation (Schwarz and Bissell, 1978) and the percentage of collagen synthesis. This behavior, while more rapid than in vivo, does accurately mimic the symptoms of the disease scurvey (Barnes, 1975). Therefore, the response to this parameter by PAT cells in vivo and in culture are similar, if not identical. Below, we will relate other responses of PAT cells in culture to the needs of the intact organism. Our focus will be on growth potential, collagen synthesis as a developmental program, and ageing.

0 0 0 0 5 1 0 / 8 0 2

Organisms which show determinate growth potential must restrict the number of cells produced. Probably, in an avian system, this restriction is most critical because the balance between the ability to fly and adult size leaves very little room for error. PAT cells show two types of inhibition with respect to restrictions on cell division. One is the typical density dependent growth control (or cell shape control [Folkman and Moscona, 1975]), whereby normal cells lengthen their generation time as the cell density increases. The second type is an adaptation to and a neutralization of the growth promotor, in this case serum, after several generations. With PAT cells, the cell number where density dependent growth inhibition sets in, decrease after each subculture. The net result is that the number of cell divisions attainable is finite. The variations in growth potential (number of generations that can be obtained) is a function of the batch of serum and serum concentration.

In this regard, one might expect cells to achieve varying numbers of generations depending on the particular growth promotor used. This type of response was clearly demonstrated by Rheinwald and Green (1977) where keratinocytes would grow for 50 generations in medium with serum alone but would continue for another 100 generations if epidermal growth factor was additionally added. With PAT cells other growth promotors which are perhaps more neutral to the expression of differentiated function, may show promise in allowing us to extend the number of subculturings while maintaining the embryonic state of the cells (Cahn and Cahn, 1966).

This response of PAT cells to growth promotors appears to be similar to that described by Hayflick (1965) with human lung cells which showed a finite growth pattern of approximately 50 generations using 10% serum. The higher number of generations achieved in those results is probably due to the use of higher levels of serum. To adapt and neutralize the growth promoting potential of this level of serum may require more generations than with

in a medium which contains 50-fold less serum. If our analysis of this phenomenon is correct, then this limited growth potential is not an ageing phenomenon, but instead, a necessary response used to restrict the size of the organism by limiting the number of cell divisions.

The second point we wish to address, deals directly with development. Specifically, we are concerned with speed of the developmental process. With chickens, from egg to baby chick takes 21 days; the rest of growth to mature chicken requires a year (Timiras, 1972). One can understand the need for such accelerated growth in early stages of life in terms of the survival of the organism. In the simplest terms, the stage which can best survive independently against the external environment is the adult chicken: the faster the embryo becomes an adult the greater its chance for survival. Of course, factors, such as parental care, can moderate the need for speed in becoming an independent organism. However, in the chicken this basic strategy places strict requirements on the cells which are concerned with structural proteins.

To acheive this kind of accelerated developmental pattern in a tendon, requires the cells to produce a peak of collagen synthesis followed by a slow decline. This is what is observed experimentally. In early chick development, Diegelmann and Peterkofsky (1972) have shown for a similar tissue, frontal bone cells, there is a rapid turn on of collagen synthesis around day 11 (_in ovo_) which reaches a maximum level by day 13 and continues at this level for as long as they measured (day 16). With tendon cells, we know that they will make 30% collagen from day 14 to day 17 (Schwarz _et al._, 1976; Dehm and Prockop, 1972). In this paper, we show that by six months after hatching, this drops to 18% and at the adult stage the level drops to less than 1%.

The regulation of collagen production has to be under precise control for tendons too large or too small are of little use to the chicken. Our

0 0 ␣ 0 5 1 0 / 8 0 3

results from culturing PAT cells, correlate well with the idea that maintain-taining cells at high density triggers the necessary decline in collagen synthesis. The result is complicated by the fact that depending on several factors the duration of high collagen synthesis (the "C" period, Fig. 1) is variable (Schwarz and Bissell, 1977). However, the exact mechanism which controls the level of collagen synthesis needs to be deciphered because this is a critical process of development.

Our results may shed light on one other biological phenomenon. This is the observation that most organisms age or more precicely show senescence: the increasing probability of death with advancing age. While many theories of ageing exist (Timiras, 1972; Comfort, 1964), especially at the molecular level, (Kirkwood, 1977), they tend to ignore some of the unique and essential features of this phenomenon. In this regard Williams (1957) has described the basic paradox of ageing that "after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed". Thus, the problem of ageing is one of understanding the inadequacies which develop in an organism after it switches from the embryonic to the maintenance state of the adult. The extent of these inadequacies can cause the life span to be a few days or over a century, depending on the organism. Part of the problem with the maintenance state is known and is due to the fact that this stage is under only limited evolutionary selection: "For organisms in which maximum life span is known with any certainty the age structure of wild populations indicates that death from old age almost never occurs" (Williams, 1966; Comfort, 1964). One can conclude that a gene which improves the maintenance state of the organism would have no selective advantage insofar as it extends the life span. Taken together these two points lead one to predict that the maintenance state of an organism is incapable of sustaining function

indefinitely and this problem has never been selected against.

Our experiments with tendon cells appear to shed light on the deficiency of the maintenance state. In an organism like a chicken, with determinate growth, many functions of many cells have to limit the expression of products which would increase the size of the organism. As we have discussed above, tendon cells of the chicken in vivo show two states. A broad development state lasting about a year where collagen synthesis is basically high and an adult maintenance state where collagen synthesis is extremely low. We infer from our cell culture studies (Fig. 3) that a change to a maintenance state is essentially irreversible (unless, possibly, the environment of the cell were to change drastically). Therefore, the adult tendon cell, confined to making less than 1% collagen, would be limited in its ability to repair damage. In actuality, tendon damage is very slow to repair (Smith, 1966). This inability to renew by retracting developmental pathways in many tissues with many functions would lead to a slowly increasing probability of dying-senescence.

With regard to collagen and ageing one more point needs to be made. To limit growth, collagen production needs to be regulated precisely. However, a commensurate drop in cross-linking enzymes need not be under such strict control. This may account for why it has been observed that as an organism ages, collagen tends to become increasingly cross-linked (Gross, 1961; Sinex, 1966).

Assuming the above theory to be correct, one would predict that an organism which continues to grow would show less senescence. Again, using tendons as an example, continued growth would allow increased synthesis of collagen in the maintenance state consequently better ability to repair. Due to wide biological diversity, such animals indeed exist. Bidder (1932) first observed in the 1920s' that certain fish continue to grow throughout their lives and do not show senescence. These fish still die, but

0 0 0 0 5 1 0 7 8 0 4

death is due to many causes, and is roughly independent of age. He concluded "that senescence is the after-result of the mechanism which secures specific size". The actual mechanism has not been elucidated.

We conclude that senescence is a consequence of the need to find a simple solution to the requirements for both rapid development and determinate growth. That is to create a developmental state of intense production and a maintenance state where cells have lost their ability to synthesize high levels of certain products. The lack of flexibility of cells in the maintenance state leads to senescence. The actual length of the life span would be a result of how complex and fine the controls are in the maintenance state.

REFERENCES

Barnes, M.J. (1975). Function of ascorbic acid in collagen metabolism. Ann N.Y. Acad. Sci. 258, 264-277.

Bassham, J.A., Bissell, M.J., and White, R.C. (1974). Quantitative tracer studies of metabolic dynamics of animal cells growing in tissue culture. Anal. Biochem. 61, 479-491.

Bidder, G.P. (1932). Senescence. Brit. Med. J. ii: 583-585.

Bornstein, P., von de Mark, K., Wyke, A.W., Erlich, A.P., and Monon, J.M. (1975). Characterization of the pro- 1 chain of procollagen. J. Biol. Chem. 247, 2808-2813.

Cahn, R.D. and Cahn, M.B. (1966). Heritability of cellular differentiation: clonal growth and expression of differentiation in retinal pigment cells *in vitro*. Proc. Natl. Acad. Sci. USA 55, 106-114.

Comfort, A. (1964). Ageing: The biology of senescence. Holt, Rinehart and Winston, Inc. N.Y.

Daniel, C.W. (1972). Ageing of cells during serial propagation *in vitro*. In "Advances in Gerontological Research". Vol. 4, pp. 167-199. Academic Press, N.Y.

Davidson, E.H. (1964). Differentiation in monolayer tissue culture cells. Advac. Genet. 12, 143-280.

Dehm, P. and Prockop, D.J. (1971). Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon. Biochem. Biophys. Acta. 240, 358-369.

Diegelmann, R.F. and Peterkofsky, B. (1972). Collagen biosynthesis during connective tissue development in chick embryo. Dev. Biol. 28, 443-453.

Folkman, J. and Moscona, A. (1978). Role of cell shape in growth control. Nature 273, 345-349.

Gross, J. (1961). Ageing of connective tissue; the extracellular components. In "Structural Aspects of Ageing". (G.H. Bourne, ed.) Hafner Publishing Co., N.Y.

Ham, R.G. (1965). Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. USA 53, 288-293.

Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37, 614-636.

Kirkwood, T.B.L. (1977). Evolution of ageing. Nature 270, 301-304.

Peterkofsky, B. and Diegelmann, R. (1971). Use of mixture of protenase-free collagenase for specific assay of radioactive collagen in the presence of other proteins. Biochem. 10, 988-994.

Peterkofsky, B. (1972a). The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. Arch. Biochem. Biophys. 152: 318-328.

Peterkofsky, B. (1972b). Regulation of collagen secretion by ascorbic acid in 3T3 and chick embryo fibroblasts. Biochem. Biophys. Res. Commun. 49, 1343-1350.

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.

Peterkofsky, B. and Prather, W. (1977). Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. J. Cell Physiol. 90, 61-70.

Rheinwald, J.G. and Green, H. (1977). Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265, 421-424.

Schwarz, R., Colarusso, L., and Doty, P. (1976). Maintenance of differentiation in primary cultures of avian tendon cells. Exp. Cell Res. 102, 63-71.

Schwarz, R.I. and Bissell, M.J. (1977). Dependence of the differentiated state on the cellular environment: modulation of collagen synthesis in tendon cells. Proc. Natl. Acad. Sci. USA 74, 4453-4457.

Schwarz, R.I. and Bissell, M.J. (1978). Full hydroxylation of proline may be necessary but is not sufficient for a high level of collagen synthesis in primary avian tendon (PAT) cells. Federation Proc. 37, 152a (abstract).

Sinex, F.M. (1968). The role of collagen in aging. In "Treatise on Collagen". (B.S. Gould, ed.) Vol. 2b, pp. 409-444. Academic Press, N.Y.

Smith, M.J. (1966). Theories of aging. In "Topics in the Biology of Aging". (P.L. Krohn, ed.). Interscience Publishers, N.Y.

Timiras, P.S. (L972). Developmental Physiology and Aging. MacMillan, N.Y.

Todaro, G.T. and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17, 299-313.

Williams, G.C. (1957). Pleiotrophy, natural selection, and the evolution of senescence. Evolution 11:398-411.

Williams, G.C. (1966). Adaption and natural selection. p. 226. Princeton University Press, Princeton, N.J.

0 0 0 0 5 1 0 7 8 0 6

FIGURE LEGENDS

Fig.1. Collagen synthesis by PAT cells initially seeded at 1.2×10^6 over an 8-day period in medium F12 supplemented with 0.5% fetal calf serum and 50 ug/ml ascorbic acid. The left ordinate shows the percentage of ^{3}H proline sensitive to collagenase; the right ordinate is this value corrected for the ratio (5.2) of proline content in collagen to that in other cellular proteins (correction based on the method used by Diegelmann and Peterkofsky [1972]). Only the corrected value is referred to in the text. The curve has been divided up into 4 distinct periods with cross-overs based on whether the cells are producing 20% collagen.

Fig. 2. The effect of various serum concentrations on the collagen synthesis of PAT cells at the end of the week. Cells were grown in F12 medium supplemented with 50 μg/ml ascorbic acid, and were seeded at 0.8×10^6 cells/flask. The serum concentrations tested were 1% (●), 0.5% (■) and 0.25% (▲). The fact that the rise in collagen occurs later in the week in this experiment than in the one reported in Fig. 1 is due to the lower initial inncoulation of cells.

Fig. 3. Level of collagen synthesis of secondary cultures of PAT cells made on day 4 (□) and on day 7 (○). In both cases the collagenase, trypsin, pancreatin solution (see Methods) used to dissociate the cells from the flask was left on 1 hr. While 1 hr was excessive for subconfluent cultures, it was necessary for confluent monolayers. The primary culture was seeded at 0.8×10^6 cells/flask; secondary cultures were seeded at 0.6×10^6 cells/flask. All cultures were grown in F12 medium supplemented with 0.2% fetal calf serum and 50 ug/ml ascorbic acid.

Fig. 3. (cont'd)

The fact that the collagen synthesis of secondary cultures made on day 4 closely follows the primary cultures is a result of three factors: the low splitting (approximately 1:2); the density dependent inhibition of growth by primary cultures which occurs at the end of the week; and the fact that secondary cultures seeded at this density have little or no lag before exponential growth.

Fig. 4. PAT cells in primary (●), secondary (○), and tertiary (□) cultures. Primary cells were seeded at 0.6×10^6, secondary and tertiary cells at 0.2×10^6 cells/flask. Primary cultures were split to secondary cultures on day 5; secondary cultures were split to tertiary cultures on day 9. The medium was F12 supplemented with 0.2% serum and 50 ug/ml ascorbic acid.

a) Shows the percentage of collagen synthesis.

b) Shows growth curves.

ACKNOWLEDGEMENT

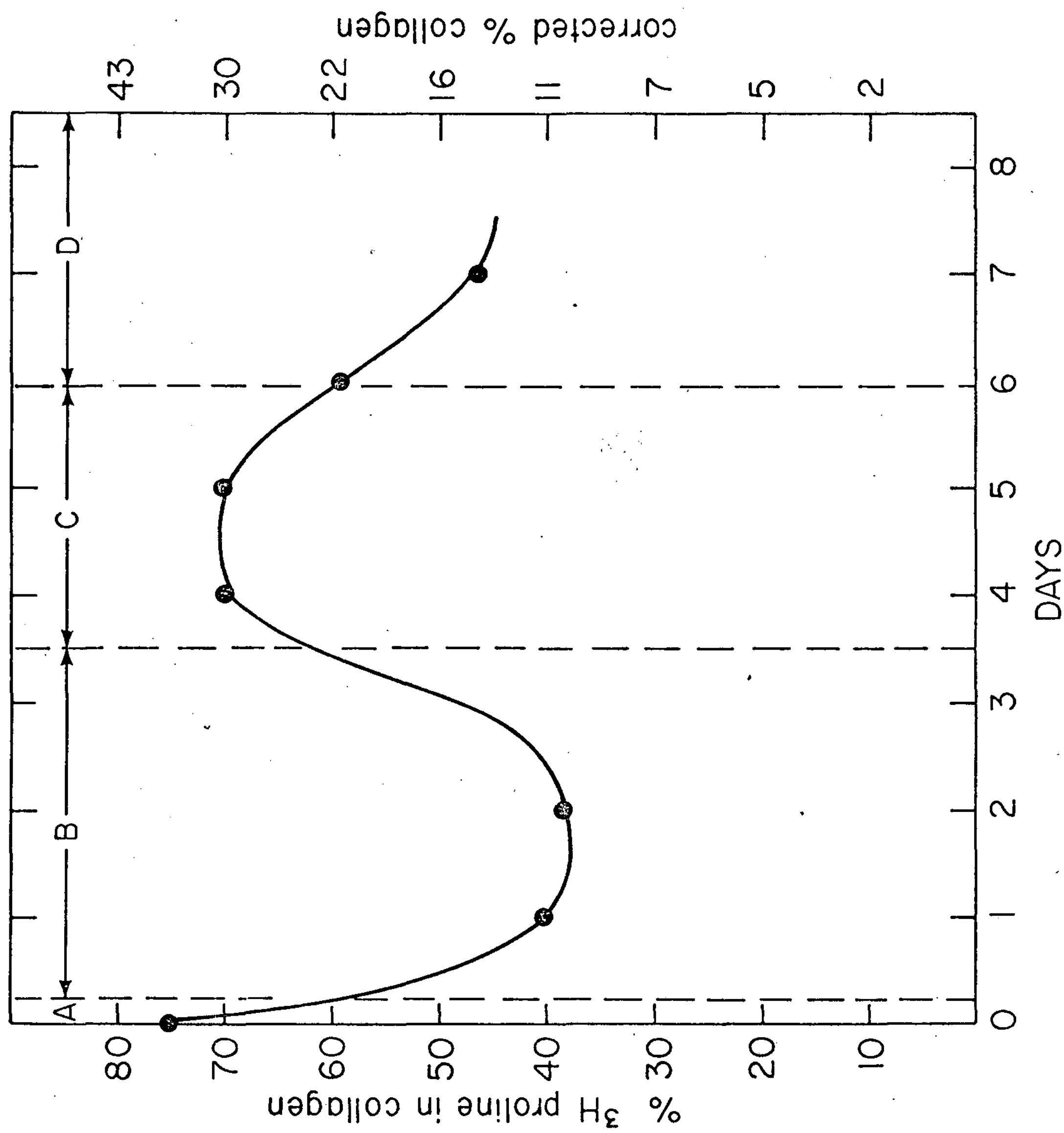
R.I.S. would like to thank two people for major help with the discussion: Dr. Steve Gaulin for correcting basic biological deficiencies and for pointing me in the right direction and Dave Krohne for helping me to decipher biological facts from fantasies.

This investigation was supported in part by the National Science Foundation (grant No. PCM 77-14982); in part by the Division of Biomedical and Environmental Research of the Department of Energy; and by a National Institutes of Health Fellowship 1F32 CA 05807-01, from the National Cancer Institute to R.I.S.

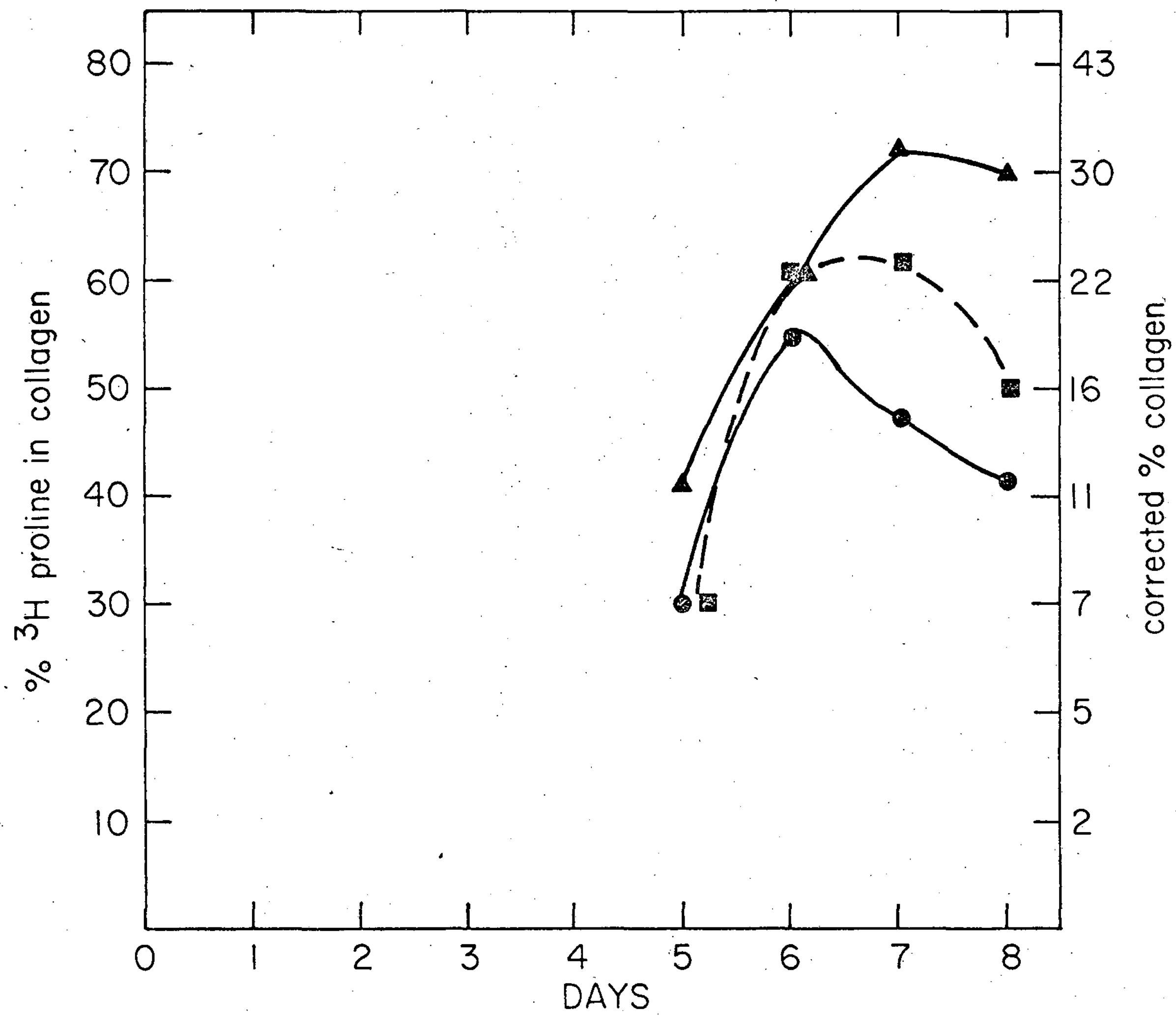
Table 1

	16 day embryo tendons	< 6 month chicken tendons	∿ 5 year chicken tendons
Corrected % collagen synthesized	31-34%	18%	0.4-0.9%

The corrected percentage collagen used in this table is based on an adjustment for the proline content in collagen to the average protein which is described in the legend to Fig. 1. In addition, we have assumed that in fully hydroxylated collagen, 47% of the prolines are hydroxylated (Bornstein, et al., 1972). Each sample was analyzed in duplicate and averaged; the range of values reflects variation between samples.

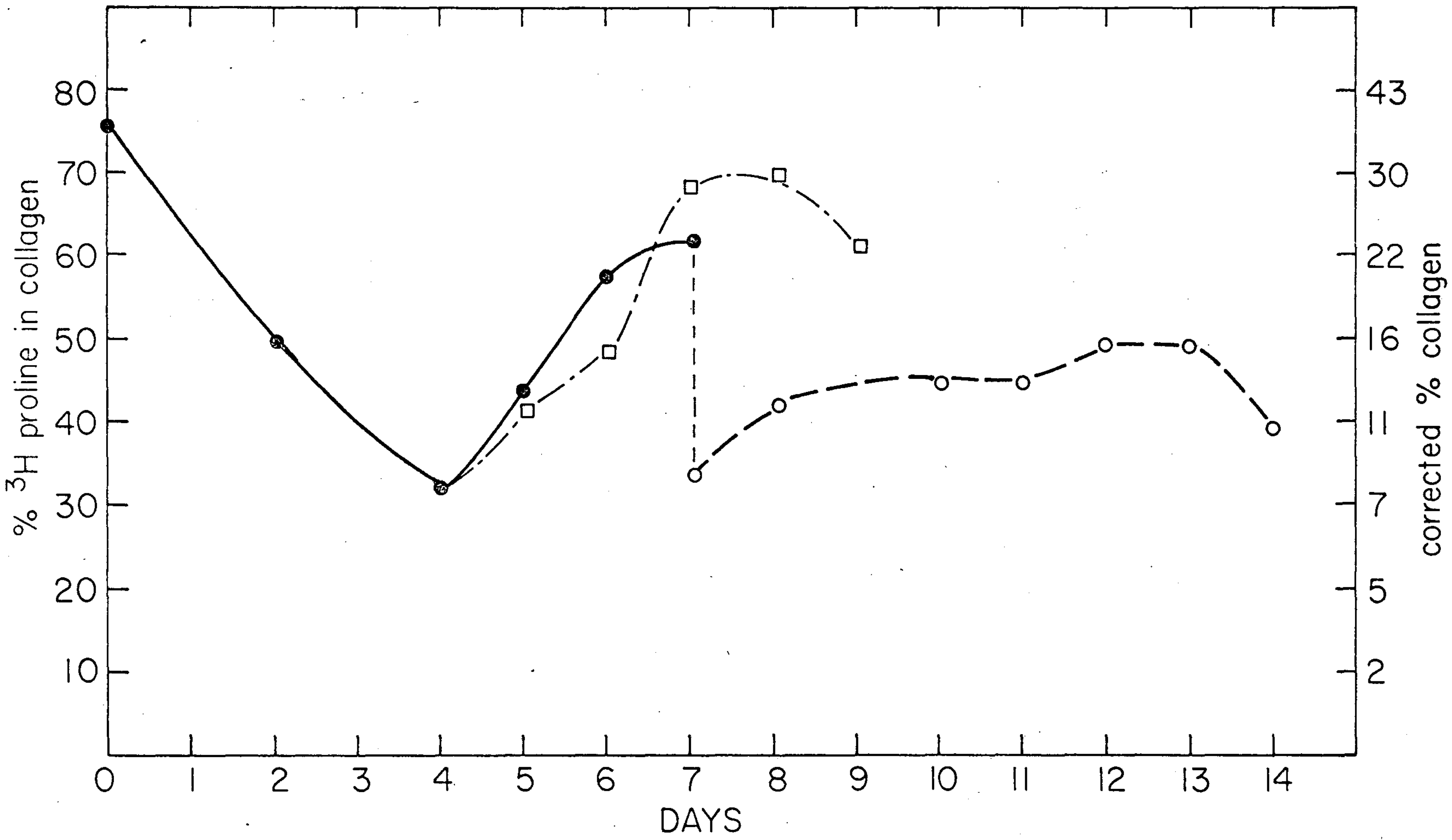


Schwarz et al. Figure 1

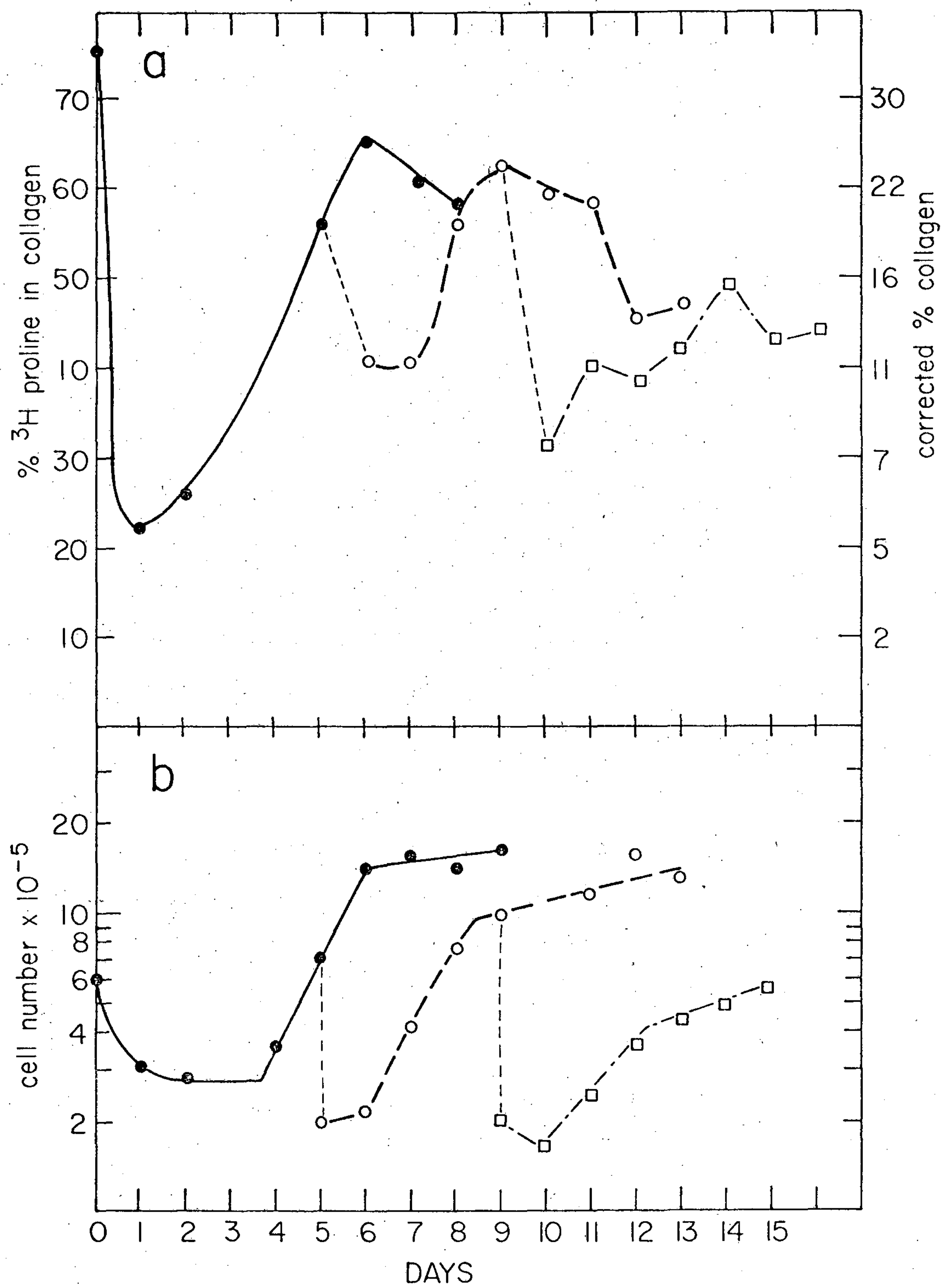


Schwarz et al. Figure 2

0 0 0 0 5 1 0 7 8 0 9



Schwarz et al. Figure 3



Schwarz et al. Figure 4

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