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STUDIES ON GUINEA PIG SKIN CELL CULTURES

VI. *Growth Kinetics of Epidermal Keratinocytes and Dermal Fibroblasts*

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Abstract. The growth kinetics of epidermal keratinocytes (EK) and dermal fibroblasts (DF) have been determined by four different methods: incorporation of ^{3}H-thymidine into DNA (^{3}H-μg DNA ratio); ^{3}H-thymidine/^{14}C amino acid incorporation ratio (^{3}H : ^{14}C ratio); ^{3}H-thymidine labelled nuclei, and colchicine-blocked metaphase counts. The growth curve of EK was no different when plotted with the ^{3}H : ^{14}C ratio than with the ^{3}H-μg DNA ratio. However, this was not true for DF. The replacement of sodium bicarbonate with Hepes buffer in the culture medium did not greatly affect the shape of the EK growth curve, whereas the DF growth curve became diphasic instead of monophasic. The elimination of mature (differentiated) keratinocytes from the very onset of EK culture had a profound effect on the EK growth curve. DNA synthesis peaked at day 1 in cultures without, instead of day 9 in cultures with differentiated cells. Furthermore, mitotic activity did not show up before day 6. This suggests that (i) EK in culture are sensitive to the G1 inhibitor released by differentiated epidermal cells, and (ii) they remain in G2 for about 5 days. Thus, EK in culture seem to continue to be susceptible, as in vivo, to homeostatic regulation through the action of G1-G2 inhibitors.

Key words: Skin cell culture; Epidermal keratinocytes; Dermal fibroblasts; Growth kinetics

In the first paper of this series it was shown that the growth curves of epidermal keratinocytes (EK) and those of dermal fibroblasts (DF) were both monophasic, but differed as regards the date of the peak, which was 9 days for keratinocytes and 5 days for fibroblasts (11).

However, the method of evaluating growth capacity, based on the incorporation ratio of ^{3}H-thymidine to ^{14}C-amino acid mixture, could be criticised on the grounds that it was not necessarily representative of the actual growth, since the incorporation of amino acids corresponds to the synthesis of both structural (mitosis) and specific (differentiation) proteins.

Furthermore, since the time of this first publication, two improvements in technique have led to a new method of culture. First, the pH has been stabilized through the use of Hepes buffer *ad modum* Ceccarini & Eagle (2). Second, in order to select only those cells which are capable of mitotic activity in vivo, differentiated keratinocytes which do not grow in culture (12) have been discarded.

In the present paper, several methods have been used to evaluate the growth capacity of the cultures. First we employed the incorporation ratio of ^{3}H-thymidine to ^{14}C-amino acid mixture, as already published (11). Second, we estimated DNA synthesis by measuring the amount of thymidine incorporated into cellular DNA. Third, we counted the number of ^{3}H-thymidine labelled cells per surface area. Fourth, we counted the number of colchicine-blocked metaphases, and fifth, we considered the density of silver grains over nuclei in autoradiographed cultures at day 1 and at day 14.

It will be shown (i) that the growth kinetics of epidermal keratinocytes can be expressed equally well by the incorporation ratio of ^{3}H-thymidine to ^{14}C-amino acids, and by the ratio of ^{3}H-thymidine dpm to μg of DNA (a procedure which does not apply to fibroblasts); (ii) the elimination from the cultures of differentiated keratinocytes allows basal cells to move into S phase, and (iii) epidermal keratinocytes cultured in the absence of in vivo differentiated cells enter mitosis after a block in G2 phase of about 5 days.

MATERIALS AND METHODS

A. *Cultures*

Cells have been cultured in three different conditions.

(1) *Condition 1* represents the first, "old" method of culture, published in the first paper of this series (11).

Epidermal keratinocytes and dermal fibroblasts were isolated as described previously. They were seeded at 250000 cells per sq.cm on coverslips placed in flattened tubes. BME supplemented with 10% calf serum and buffered with sodium bicarbonate was used as growth medium.

(2) *Condition 2* represents the second, "new" method of culture, the principle of which was published in 1976 (10).

In this method the differentiated keratinocytes which remained attached to the steristrip were discarded. Only the basal cells which remained attached to the dermis were harvested, by shaking the piece of dermal tissue in a hemolysis tube containing medium.

Epidermal keratinocytes and dermal fibroblasts were respectively seeded at 500 000 and 50000 cells per sq.cm in (75 cm^2) Falcon flasks or (30 mm) Falcon Petri dishes. BME supplemented with 10% fetal calf serum and buffered with Hepes was used as growth medium.

(3) *Condition 3* represents a combination of conditions 1 and 2. Namely: the pH was controlled as in condition 1 (sodium bicarbonate) and differentiated cells were eliminated as in condition 2.

B. *Evaluation of cell growth*

1. *Methods based on the incorporation of ^3H-thymidine*

Three methods have been employed. In the first the cultures were pulsed simultaneously with ^3H-thymidine and ^{14}C-amino acids, and growth capacity was expressed as the ratio of ^3H : ^{14}C cpm.

In the second, cultures were pulsed with ^3H-thymidine as in the first method, and DNA synthesis was used to assess growth capacity. It was expressed as the ratio of ^3H dpm to μg of DNA measured by the technique of Burton.

In the third, the cultures were also pulsed with ^3H-thymidine and the incorporation was revealed by autoradiography.

Technically three procedures have been followed.

Procedure 1. EK and DF cultures in flattened tubes were pulsed every other day with 1 μCi/ml of methyl ^3H-thymidine (spec.act.: 14.9 Ci/mM, CEA, Saclay, France) and 1 μCi of ^{14}C-amino acid mixture (spec.act.: 30 mCi/milliatom of C; CEA, Saclay, France) for 30 minutes.

The coverslips were processed for radioactivity measurement according to the direct counting technique of Fallot et al. (7). The growth capacity of the cell population was expressed as the ratio of ^3H : ^{14}C cpm.

Procedure 2. In procedure 2, methods 1 and 2 have been applied to the same populations of cultured cells.

One flask of EK and one of DF were incubated daily in a fresh medium containing 1 μCi/ml of ^3H-thymidine (spec.act. 40 Ci/mM; Amersham) and 1 μCi/ml of ^{14}C-amino acids (spec.act. 25 mCi/milliatom of C; Amersham) for 1 hour. The cells were then washed three times, dissolved in 1 N NaOH, neutralized in HCl and precipitated in PCA. The detailed treatment of PCA precipitates has been published elsewhere (4). Briefly, they were hydrolysed in hot PCA and centrifugated. One aliquot of the supernatant was used to measure the radioactivity in a liquid scintillation counter. Two other aliquots were used to measure the amount of DNA by the diphenylalanine method of Burton.

After hydrolysis in PCA, the pellet was hydrolysed in 6 N HCl overnight. An aliquot of the hydrolysate was counted for radioactivity. The total radioactivity was estimated by adding the ^{14}C dpm in PCA to the ^{14}C dpm in HCl.

The growth capacity of the cell population was expressed by the ratio ^3H dpm/μg of DNA (DNA synthesis) and by the ratio ^3H dpm/^{14}C dpm, as in procedure 1.

Procedure 3. Autoradiography: Every day, one Petri dish of EK and one of DF were incubated in fresh medium containing 1 μCi/ml of ^3H-thymidine for 1 hour. The Petri dishes were then washed with saline, fixed with 1:3 glacial acetic acid/methanol or 10% formaldehyde, rinsed and air dried. The Petri dishes were dipped with Ilford L4 (diluted 1:4) and stored in the dark at 4°C for 1 week. After development they were stained with Giemsa for 1 hour or Toluidine Blue for 5 minutes. Labelled cells were counted on a total surface area of 5.6 sq.mm per dish, representing a rectangle of 32 mm long by 0.175 mm wide stretched diametrically across the Petri dish. A nucleus was considered labelled when it was covered by 5 or more grains.

2. *Methods based on the evaluation of mitotic activity*

Mitotic counts. Every day one Petri dish of EK and one of DF were incubated in fresh medium containing 40 μg/ml of colchicine for 3 hours. They were washed, fixed, rinsed, stained with Giemsa and air dried. The numbers of mitotic figures were counted on a surface of 5.6 sq.mm, as described above (see Procedure 3).

Autoradiographic grain density. The day after plating, two series of twenty Petri dishes were incubated in fresh medium containing 1 μCi/ml of ^3H-thymidine for 1 hour. The cells were then washed five times with cold medium, and refed with fresh medium. Every day one Petri dish of EK and one of DF were fixed and processed for autoradiography. The density of grains was estimated high when grains could not be counted and low when grain count was feasible. The surface occupied by nuclei at days 1 and 14 has been evaluated indirectly: photographic pictures were taken, representative series (over 150) of nuclei pictures were cut out, photographic paper cuts were weighed and the difference in weights was statistically calculated in a χ^2-test. A decrease in grain density (from high to low) without increase in nuclear surface was expected to reflect mitotic activity through progressive dilution after each cell doubling of the radioactive label.

RESULTS

A. *Evaluation of growth capacity and DNA synthesis*

(1) *^3H : ^{14}C ratio in cells cultured according to condition 1.* The growth curves of EK and DF cultured according to the old method (sodium bicarbo-

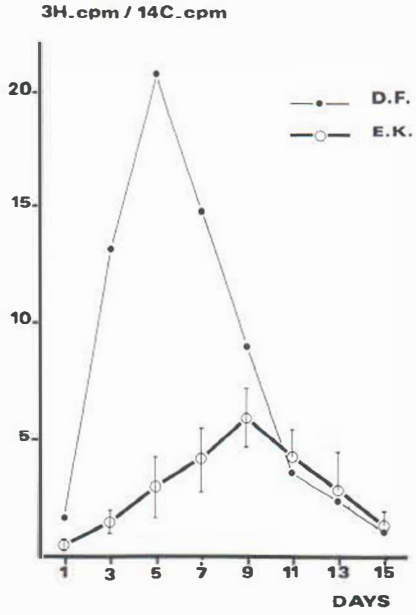

Fig. 1. Growth curves of epidermal keratinocytes (EK), 10 experiments, and dermal fibroblasts (DF) one experiment, grown according to Condition 1 (see Methods). The growth capacity is expressed by ^{3}H-thymidine to ^{14}C-amino acid incorporation ratio. The two curves are monophasic. EK peaks at day 9 and DF at day 5.

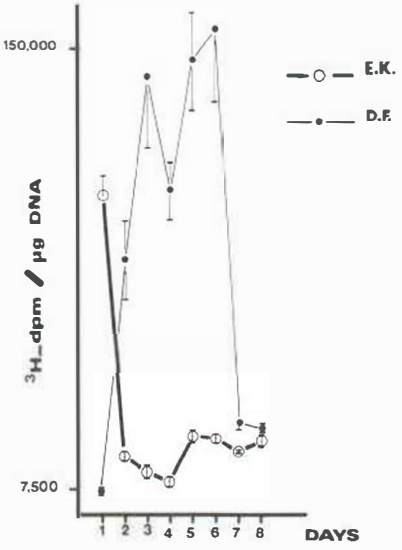

Fig. 2a. Growth curves of EK (10 experiments) and DF (10 experiments) grown according to Condition 2 (see Methods). The growth capacity is expressed by the ratio ^{3}H-thymidine to µg DNA. The two curves are biphasic. EK shows maximum DNA synthesis at days 1 and 5; DF at days 3 and 6.

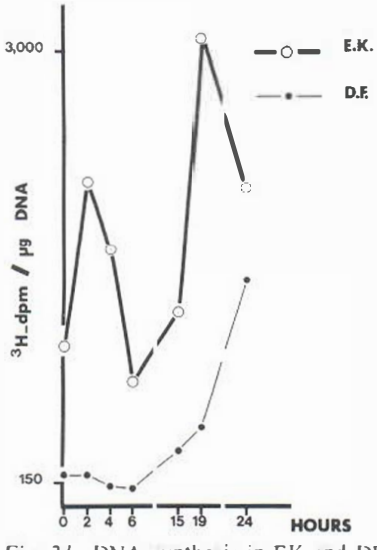

Fig. 2b. DNA synthesis in EK and DF (one experiment each) during the first 24 hours of culture. EK cells show 2 early peaks: the first at 2 hours and the second at 19 hours. There is no early peak in DF culture.

nate buffer+differentiated cells) are shown in Fig. 1. Each point of the EK curve represents the mean value of 10 experiments. Growth capacity is expressed as the cpm ratio of ^{3}H-thymidine to ^{14}C-amino acids (abbreviated: ^{3}H : ^{14}C ratio). The ^{3}H : ^{14}C ratio increased steadily from day 1 to reach a peak at day 9. There was but one peak during the 2 weeks of the study.

Similarly the DF curve exhibited only one peak throughout the lag period. The main difference between the two curves is in the dates of the two peaks.

(2) *^{3}H : µg DNA ratio in cells cultured according to condition 2.* The growth curves of EK and DF cultured according to the new method (Hepes buffer+elimination of differentiated cells) are shown in Fig. 2*a*. Each point of the curves represents the mean value of at least 10 experiments. DNA synthesis is expressed as the ratio of ^{3}H-thymidine dpm to µg of DNA (abbreviated: ^{3}H : µg DNA ratio). In EK cultures, the ^{3}H : µg DNA ratio is highest at day 1. In fact, maximal incorporation of thymidine into DNA may happen earlier, as shown in Fig. 2*b* representing DNA synthesis during the first 24 hours in one experiment. It will be noticed that a first "wave" of DNA synthesis takes place at 2 hours of culture, i.e. very soon after attachment of the cells. Back to Fig. 2*a* it can be seen that past the first 24 hours, the lowest point of the curve is at

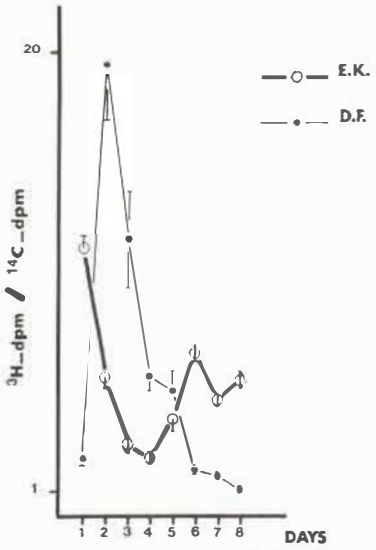

Fig. 3. Growth curves of EK (10 experiments) and DF (10 experiments) grown according to Condition 2 (see Methods). The growth capacity is expressed by ^{3}H-thymidine to ^{14}C-amino acid incorporation ratio. The curve of EK is biphasic, as in Fig. 2. The curve of DF is monophasic, with a peak at day 2.

day 4. From day 5 to 8 the level of DNA synthesis is distinctly above the trough of day 4.

In DF cultures there are two clearly identifiable peaks—one at day 3 and the second at day 6. This second peak is followed by a sharp fall which brings the curve down to slightly above the level of day one.

(3) ^{3}H : ^{14}C *ratio in cells cultured according to condition 2.* In this series of 10 experiments for each culture (Fig. 3), EK and DF have been cultured according to the new method, as in Fig. 2*a*. The growth capacity has been estimated by plotting the ^{3}H : ^{14}C ratio, as shown in Fig. 1. Grossly speaking, the growth curve of EK is similar to that shown in Fig. 2*a*. On the other hand, the growth curve of DF is different. There is one peak—at day 2 instead of two peaks, on days 3 and 6 respectively.

(4) *^{3}H : μg DNA ratio in cells cultured according to condition 3.* In this series of experiments (Fig. 4) one culture of EK and one culture of DF were made in sodium bicarbonate buffered medium (as shown in Fig. 1) plus elimination of differentiated cells (as in Fig. 2*a*).

The shape of the EK curve does not differ greatly from that of EK cells in Fig. 2*a*. In particular, DNA synthesis is high at the beginning of the culture. Some changes can be noted, however. DNA synthesis is highest at day 2 instead of day 1, and restoration of DNA synthesis after the trough does not seem to happen as clearly as in Fig. 2*a*. As compared with the EK curve in Fig. 1, the main difference is the precocity of the peak of DNA synthesis, which occurs at day 2 instead of day 9.

As regards the curve of DF, it shows one peak—at day 5, as shown in Fig. 1.

(5) *Labelled nuclei counts in autoradiographed cultures made according to condition 2.* Results are shown in Fig. 5*a*.

The growth curve of EK is very similar to that shown in Fig. 2*a*. Similarly, the curve of DF, though not identical, has the same shape as that shown in Fig. 2*a*. In particular there are two peaks during the first 6 days and a drop at day 7.

B. *Evaluation of mitotic activity*

(1) *Colchicine-blocked metaphase counts.* Results of counts are plotted on Fig. 5*b*.

Mitotic activity in EK was not detectable until day 6. A sharp rise in metaphase counts occurred from day 6 to day 8. No counts were made after day 8.

In DF, mitotic activity was noted throughout the

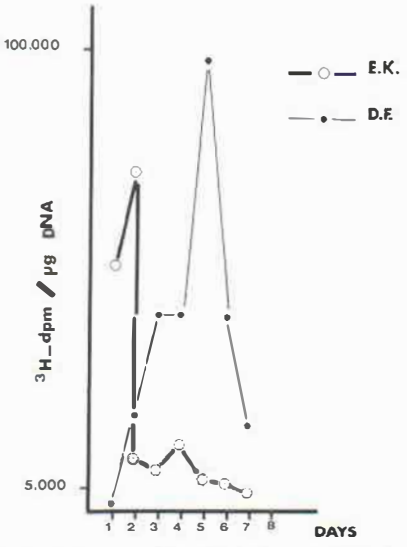

Fig. 4. Growth curves of EK (one experiment) and DF (one experiment) grown according to Condition 3 (see Methods). The growth capacity is expressed by the ratio ^{3}H/μg DNA. The curve of EK is biphasic and resembles that of Figs. 2 and 3. The curve of DF is monophasic, with a peak at day 5.

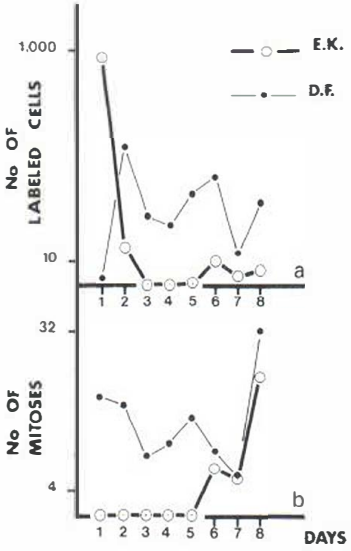

Fig. 5. Comparison of EK and DF growth kinetics as estimated by the counts of ^{3}H-thymidine labelled cells in autoradiographed cultures (5*a*) and mitotic counts (5*b*) per surface area of 5.6 sq.mm. Cells were cultured according to Condition 2 (see Methods). Labelled cells curves (5*a*) grossly parallel the curves of DNA synthesis shown in Fig. 2*a*. No mitosis (5*b*) was seen in EK before day 6. There was a sharp rise thereafter. In DF, mitotic activity was recorded throughout the 8 days of the experiment. High value at day 8 raises the question of contamination of DF by hair follicle epidermal cells (see Discussion). The comparison of 5*a* with 5*b* suggests that EK are blocked in G2 during the first days of culture.

culture from day 1 to 8. Highest values were recorded at day 8.

(2) *Autoradiographic grain densities.* The density of autoradiographic silver grains was very high in cultures aged 1 day and low in cultures aged 14 days. The surface of 260 nuclei at day 1 and that of 176 nuclei at day 14 have been compared in a χ^2-test. The surface of 14 day nuclei was found significantly greater by an order of magnitude of 2+ ($p<0.001$).

DISCUSSION

1. *Growth kinetics of EK*

The growth curve shown in Fig. 1 has been obtained in plotting the ratio of ^{3}H-thymidine to ^{14}C-amino acid incorporation (^{3}H : ^{14}C ratio) by EK maintained in a culture medium buffered with sodium bicarbonate and without eliminating in vivo differentiated cells from the seed suspension. This curve has a monophasic shape with a peak at day 9.

The growth curve shown in Fig. 2*a* was obtained by plotting the amount of ^{3}H-thymidine incorporated into cellular DNA (^{3}H : μg DNA ratio) by EK maintained in a culture medium buffered with Hepes and after elimination of in vivo differentiated cells from the seed suspension. This curve exhibits a diphasic shape with highest values at day 1 and 5 and a trough at day 4.

The difference observed between Fig. 1 and Fig. 2*a* can be explained on three different grounds: (i) the mode of growth evaluation (^{3}H : ^{14}C ratio versus ^{3}H : μg DNA ratio); (ii) the buffer used in the culture medium (sodium bicarbonate versus Hepes) and (iii) the presence or absence in the cell seed suspension of in vivo differentiated keratinocytes.

(1) *Influence of the mode of growth evaluation.* In comparing Fig. 2*a* with Fig. 3, it can be seen that the mode of evaluation does not influence significantly the shape of the curve when EK are cultured in the same conditions.

This is interesting, because the evaluation of cellular DNA by Burton's technique requires a relatively large number of cells. On the contrary, the measure of amino acid incorporation can be achieved by using about 10 times fewer cultured cells, which makes this technique rather economical in terms of cells, and time. This might represent a first step toward the miniaturization of the evaluation of epidermal cell growth in culture, an important goal in cellular pharmacology of the skin.

(2) *Influence of the buffer.* In comparing Fig. 2*a* with Fig. 4 it can be seen that the buffer does not profoundly alter the shape of the curve. It does modify the time of the peaks but the highest DNA synthesis value is still recorded at the beginning of the culture. This means that the change in buffer does not account for the differences between Figs. 1 and 2*a*.

(3) *Influence of in vivo differentiated cells.* Since the differences between Fig. 1 and Figs. 2*a*, 3 and 4 cannot be explained on the basis of the mode of growth evaluation or the buffer, they must be due to the presence of in vivo differentiated cells in cultures of Fig. 1 and the absence of these cells in Figs. 2*a*, 3 and 4. This also applies to Fig. 5*a*.

These differences can be understood in considering the position of basal cells within the cell cycle at the time of culture inception. According to Gelfant (8), 10% basal cells are blocked in G2 and 90% are in cycle. Since the number of cells in S

phase plus that of cells in mitosis does not exceed 10%, at least 80% basal cells are in G1.

The time during which these 80% basal cells remain in G1 is dependent in vivo, upon the release by differentiated keratinocytes of a G1 inhibitor (5, 9).

It can be hypothesized that in Fig. 1, which represents cultures in the presence of in vivo differentiated cells, there is a progressive increase in the number of cells in S phase because the G1 inhibitor, present at the start of the culture, is progressively removed by culture medium changes. By contrast, in Figs. 2*a*, 3, 4 and 5*a* which represent cultures in the absence of in vivo differentiated cells, the G1 inhibitor has been eliminated before the very onset of the culture, thus allowing 80% or so of basal cells to move en bloc to S phase.

(4) *Mitotic activity*. Fig. 5*b* reveals mitotic activity from day 6 to day 8. Further studies are needed to know whether mitotic activity is a phasic phenomenon in these cultures as it is in human cells grown in organ cultures (10). The sharp decline in the density of silver grains over nuclei in autoradiographed cultures at day 14 would suggest that population doubling has actually occurred at this time. However, the increase in the surface of labelled nuclei at day 14 would also be consistent with a block in G2. That EK cells in culture can be blocked in G2 is suggested by the lag period of 5 days which elapsed before metaphases could be detected. Since the normal time spent in G2 in vivo by basal cells is estimated to between 4 hours (8) and 8 hours (3) (but not 5 days) it seems reasonable to assume that basal cells in culture are at least partially blocked in G2. This again would not be surprising in view of the finding by Elgjo et al. (6) and by Marks (9) that basal cells release a G2 inhibitor.

A picture then emerges of how adult guinea pig epidermal keratinocytes behave in culture. At the time the skin is removed from the body, some basal cells are in mitosis, some are in S phase, others are in G2 and a majority are in G1. Cells in mitosis are not detected in the early hours of culture because of the time needed for the cells to attach to the culture vessel. Cells in S phase probably pass into G2 and those which were in G2 complete their cycle through mitosis, which can be detected as early as 2–4 hours after explanting skin in vitro (1).

As regards the bulk of basal cells in G1, their progress through the cycle will depend upon the presence in the seed suspension of high level differentiated keratinocytes. If present, the move to S phase will be progressive. If absent, the move will be a rapid one. After the S phase has occurred, there is a partial block in G2, followed by a wave of mitotic divisions. Thus, homeostatic mechanisms must be taken in account in the understanding of the biology of epidermal keratinocytes in culture.

II. *Growth kinetics of DF*

There is a difference between the curve of Fig. 1, which shows one peak at day 5, and Fig. 2*a* which exhibits 2 peaks, at days 3 and 6 respectively.

These differences can be explained on two grounds. First, they could reflect the mode of growth evaluation ($^3H:^{14}C$ ratio versus $^3H:\mu g$ DNA ratio) as for EK cultures; second, they could be due to the change in buffer (sodium bicarbonate versus Hepes).

1. *Influence of the mode of growth evaluation.* In comparing Fig. 2*a* with Fig. 3, it is apparent that the mode of growth evaluation greatly influences the shape of the curve. Instead of two peaks at days 3 and 6 as in Fig. 2*a* there is only one peak, at day 2 in Fig. 3. This can be explained on the basis that when the $^3H:^{14}C$ ratio is used to plot the curve, the denominator of the ratio expresses the capacity of the cell population to synthesize both structural and specific proteins. As long as only structural proteins are made, the ratio does not differ from the $^3H:\mu g$ DNA ratio, since the synthesis of structural proteins—like that of DNA—is but an expression of population expansion. But, if large amounts of specific proteins are being synthesized, the denominator increases much faster in the $^3H:^{14}C$ ratio than in the $^3H:\mu g$ DNA ratio. As a result, peaks of DNA synthesis may be overlooked. This is probably what happened in Fig. 3.

As a consequence, the ^3H-thymidine–^{14}C-amino acid incorporation ratio cannot be used for the evaluation of the growth of dermal fibroblasts.

2. *Influence of buffer.* In comparing Fig. 2*a* with Fig. 4 it can be seen that the first peak, at day 3 in Fig. 2*a*, disappears in Fig. 4. This possibly indicates that in stable pH the cells "recover" faster than in unstable conditions.

3. *Mitotic activity.* Colchicine-blocked metaphases have been found every day from day 1 to 8. A puzzling point in Fig. 5*b* is the high mitotic activity at day 8, knowing that the last peak of DNA

synthesis is at day 6. This would mean a G2 period of 2 days, which seems rather long. Indeed, as shown in Fig. 5*a* and 5*b*, mitotic activity and DNA synthesis peak on about the same days, which indicates that the G2 period of dermal fibroblasts lasts about a few hours, but not 2 days. An explanation for this high mitotic count at day 8 could be the fact that dermal fibroblast cultures are ordinarily more or less contaminated by epidermal keratinocytes of hair follicles. Indeed, in disrupting the dermis with collagenase, a certain number of hair bulbs are dislodged and become admixed with the cell seed suspension.

Knowing from Fig. 5*b* that epidermal keratinocytes exhibit high mitotic activity at day 8, it is not ruled out that the high value of mitotic counts in dermal fibroblast cultures at day 8 reflects a certain degree of contamination with follicular keratinocytes.

ACKNOWLEDGEMENT

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