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In vitro differentiation of a homogeneous cell population—the epidermis of *Xenopus laevis*

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SUMMARY

A simple culture technique is described for obtaining homogeneous populations of differentiating epidermal cells from adult amphibian skin. This population of cells continues its normal differentiation, namely keratinization, *in vitro*. By the third day of culture more than 99·9 % of the cells in the population contain immunoreactive keratin. During the next 5–7 days of culture these cells synthesize increasing amounts of a tissue-specific keratin-like protein until this protein constitutes more than 25 % of the total extractable carboxymethylated proteins of these cells. Under the conditions described cell division does not occur during the course of differentiation. When vitamin A is added to the culture medium the rate of keratinization is decreased; when its antagonist citral is added, keratinization is accelerated. These factors influence only the rate but not the direction of differentiation.

INTRODUCTION

Most vertebrate somatic tissues are a complex mixture of cell types. Although the vast majority of cells in a tissue exhibit a histospecific differentiation there is almost always a minor cell population present that does not appear to be specialized in any overt morphological or biochemical way. These 'unspecialized' cells may be the generative basal cells that act as progenitors of the more overtly differentiated ones or they may be the ubiquitous fibroblastic cells present in most specialized organs. A truly homogeneous population of cells from either adult or embryonic tissues which continues to function and differentiate normally when grown in tissue culture would be useful for many biochemical or differentiation studies.

Populations of animal cells that appear to be morphologically homogeneous have been obtained by using various density gradient (Cutts, 1970; Weid & Bahl, 1970; Shortman, 1972) or electrophoretic cell separation techniques (Ambrose, 1965; Hannig, 1970). But separations of cells solely on the basis of size, density or surface charge cannot ensure that they all have the same biochemical phenotype.

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In this paper a simple culture technique is described that allows the routine isolation of a homogeneous epithelial cell population from primary explants of the skin of the aquatic amphibian *Xenopus laevis*. These cells undergo a spontaneous and characteristic cytodifferentiation, namely keratinization, with the synthesis of tissue specific keratin-like proteins in almost all of the cells of the population. In addition, the influence of various environmental factors on this *in vitro* differentiation has also been explored.

MATERIALS AND METHODS

Culture techniques

Cultures were initiated using a modification of the methods described by Laskey (1970). Pieces of foot web from adult frogs were cut into approximately 2 × 2 mm squares, washed thoroughly with sterile Barth X solution minus albumin (Barth & Barth, 1959) and 10–15 equally spaced fragments placed onto 20 × 30 mm glass coverslips coated with rat tail collagen (Erhman & Gey, 1956). These coverslips were then incubated in 60 mm diameter plastic tissue culture dishes (Falcon) in a minimal amount of growth medium consisting of 62% (v/v) Dulbecco's Modification of Eagle's Medium (Flow Laboratories), 28% double-glass-distilled water, and 10% Fetal Calf Serum (Flow Laboratories). This medium (designated DME) contained, in addition, sodium penicillin G, dihydrostreptomycin and gentamycin sulfate (British Schering Ltd, Slough) at a final concentration of 100 μg/ml each, amphotericin B (Squibb) at 1 μg/ml and L-glutamine at 2 mM final concentration. The medium was changed every other day. Cultures were incubated at 22° C in a water saturated atmosphere containing 95% air and 5% CO_2.

In some experiments the above culture procedure was modified in the following ways: (i) web fragments were cultured directly on the glass coverslips without collagen or, alternatively, directly on plastic tissue culture dishes (Falcon); and (ii) for cultures maintained in free gaseous exchange with the atmosphere, Leibovitz's L 15 medium (Flow Labs) replaced the Dulbecco's Modification of Eagle's Medium used in the above mentioned medium. This amino acid buffered medium is simply referred to as L 15.

Cultures established from explants of adult heart, lung, kidney and brain were set up and maintained in the same manner as described for the web cultures for which they acted as controls.

Treatment with vitamin A or citral

Vitamin A alcohol (Koch-Light Laboratories) dissolved in ethanol was added to the culture medium to give a final concentration of 25 i.u./ml vitamin A and 0·1% ethanol. Cis–trans citral alcohol (Koch-Light Labs) was also used at a final citral alcohol concentration of 0·1% in the medium for some experiments. When either of these two alcohols were used in the medium for experimental purposes, parallel control cultures contained 0·1% ethanol in their medium.

Labeling of cultures with radioactive precursors

The measurement of incorporation of radioactive precursors into epidermal cells was limited to the study of those cells which had migrated out of cultured explants (see below). Therefore, after labeling in the medium with isotope, the cultures were washed with modified Barth X solution and the explant was carefully dissected away from the substrate leaving the migrant cells attached to the substratum. These cells were either extracted for keratin-like proteins, prepared for autoradiography or dissolved for scintillation counting as described in the Results section.

The following isotopes were obtained from the Radiochemical Centre, Amersham: thymidine (methyl-^{3}H) at 20 Ci/mmol; uridine (5–^{3}H) at 25 Ci/mmol; L-cystine hydrochloride (3–^{3}H) at 2·4 Ci/mmol-reduced and used as cysteine; and ^{125}I (for protein iodination) at about 14 Ci/mg.

Tritium was counted in a Wallac (LKB) scintillation counter with a counting efficiency of between 35 and 40 %. Gamma radiation was counted in a Wallac Decem-GTL 300–500 counter.

Autoradiography

Cell sheets were fixed in ethanol:acetic acid (3:1), washed with running tap water, extracted for 1 h with cold 10 % trichloracetic acid (TCA), washed again with water and finally dipped in liquid Ilford K2 Emulsion (diluted 1:1 with water containing 1 % glycerol). Development and staining were by standard techniques.

Histochemical staining

Cells were stained by standard histochemical methods for the detection of mucin (mucopolysaccharides) using the periodic acid–Schiff (PAS) reagent. Keratin-like proteins were selectively stained by using either the mercury orange or performic acid-alcian blue methods described by Pearse (1968).

Extraction and electrophoresis of S-carboxymethyl (SCM-) and keratin-like proteins

Keratin-like proteins (KLPs) were extracted from isolated epidermal tissues and then analysed by polyacrylamide gel electrophoresis as previously described (Reeves, 1975). Isolated epidermal tissues from either cultured cell sheets or whole adult skin were extracted with buffered saline, defatted and then extracted with a medium containing 0·1 M ethanolamine, 0·1 M 2-mercaptoethanol and 8 M-urea (deionized). The extracted proteins were then carboxymethylated with 0·6 M iodoacetic acid at pH 9·5 and the resulting purified and dialyzed product run on polyacrylamide disc gels according to the methods of Davis (1964).

As reported previously (Reeves, 1975) not all of the proteins extracted from epidermis by the above procedure can be unambiguously classified as keratin-like proteins. The saline insoluble proteins that are solubilized and carboxy-methylated by this technique are thus referred to as S-carboxy-methylated or SCM-proteins. The true keratin-like proteins (bands I and II as seen on poly-acrylamide gels; see Results and Reeves, 1975) constitute, depending on the particular preparation, between 65% and 90% of the total SCM-proteins extracted from adult epidermal tissues. The fraction of total epidermal proteins extracted as SCM-proteins varies between 42% and 63% in different preparations.

Production and labeling of antibodies

The production, in rabbits, and characterization of a monospecific anti-KLP antibody preparation against one of the tissue specific keratin-like proteins (band I) found only in the epidermis of post-metamorphic animals has previously been described (Reeves, 1975).

Purified immunoglobulin preparations were labeled with either sodium iodide-125 according to the lactoperoxidase method of Marchalonis (1969) or with fluorescein isothiocyanate (Sigma Chemicals) by methods described by Clausen (1969) and Goldman (1968).

Reaction of ^{125}I-labeled antibodies with haptens

Antibody preparations against band I-KLPs were labeled to a known specific activity with ^{125}I. These iodinated antibodies were then reacted by immunodiffusion with SCM-proteins that had been extracted from cultured epidermal cells and incorporated into 1% agar as previously reported (Reeves, 1975). After removal of unreacted antibody by diffusion, the amount of radioactivity in the ^{125}I-antibody/antigen complex was counted in a gamma counter. This amount of radioactivity was related to a standard curve based on the reaction of the same labeled antibody solution to known concentrations of electrophoretically purified band I-KLPs incorporated into agar to determine the amount of this type of keratin-like protein in various SCM-protein extracts. All determinations were made under conditions of antibody excess.

All protein concentrations were determined using the method of Lowry, Rosebrough, Farr & Randall (1951) with a standard curve of bovine serum albumin for reference.

Reaction of fluorescein-labeled antibodies with cells

Web cultures to be reacted with antibody were washed with modified Barth X solution and then fixed with ice-cold acetone or ethanol (95%) for 1 h. The culture was air-dried and then washed for 1 h in running tap water. The cultures were then washed for three 15 min periods with 0·9% NaCl (w/v) solution

buffered at pH 7·2 with 0·01 M phosphate salts. The buffered saline solution was removed and a drop of fluorescein-labeled antibody (at 0·1 mg/ml of buffered saline solution) added to the culture. The reaction was allowed to occur for between 2 and 24 h in a water saturated atmosphere at 4 °C and then the unreacted antibody removed by extensive washing with the buffered saline solution. The coverslip cultures were fixed with 95 % ethanol and the coverslips mounted, cell side down, onto a clean glass slide with distilled water for observation under ultraviolet light in the fluorescence microscope.

RESULTS

Course of in vitro *differentiation*

When a web explant is cultured on collagen, plastic or glass, a coherent sheet of epithelial cells migrates out from the fragment over the substratum. The cells within this monolayer sheet are almost exclusively epithelioid and there is no apparent contamination with other cell types such as fibroblasts or melanocytes (Fig. 1 B), suggesting that the great majority of the cells within this monolayer have migrated out from the epidermal layers of the explant rather than from the more connective tissue-like dermis (Fig. 1 A).

With increasing time in culture this epithelial cell sheet slows its outward migration and begins to undergo a characteristic morphological change. By 6–8 days after initiation of a culture the sheet has stopped expanding and the cells show increasing granularity, cytoplasmic opacity, and rounding up of many cells as the entire sheet undergoes near synchronous differentiation (Figs. 1 B–D). When living cultures are viewed with polarization microscopy it is seen that as differentiation progresses the cells within the sheet become increasingly birefringent (Figs. 1 E, F). During this continuous cytodifferentiation there is considerable cell death and sloughing off of cells from the sheet into the medium until finally the entire cell sheet detaches from the substratum, rolls up, and is lost into the medium. By 10–12 days of culture there are few explants with original epithelial outgrowths left.

That this progressive cellular death is due to a normally occurring terminal differentiation of the epithelial cells and is not just a result of inadequate or toxic culture conditions leading to necrosis and degeneration, can be inferred from a number of observations. Cultures from other adult tissues such as heart, lung, kidney and brain set up under identical conditions show progressive migration and growth without any massive necrosis or cellular death and can be maintained under the same culture conditions for extended periods of time with or without subculturing. The culture conditions used are therefore adequate to support normal, non-terminally differentiating cell cultures almost indefinitely.

Further evidence that the cells in the migrating cell sheet are a terminally differentiating population has been obtained by examining their rate of division.

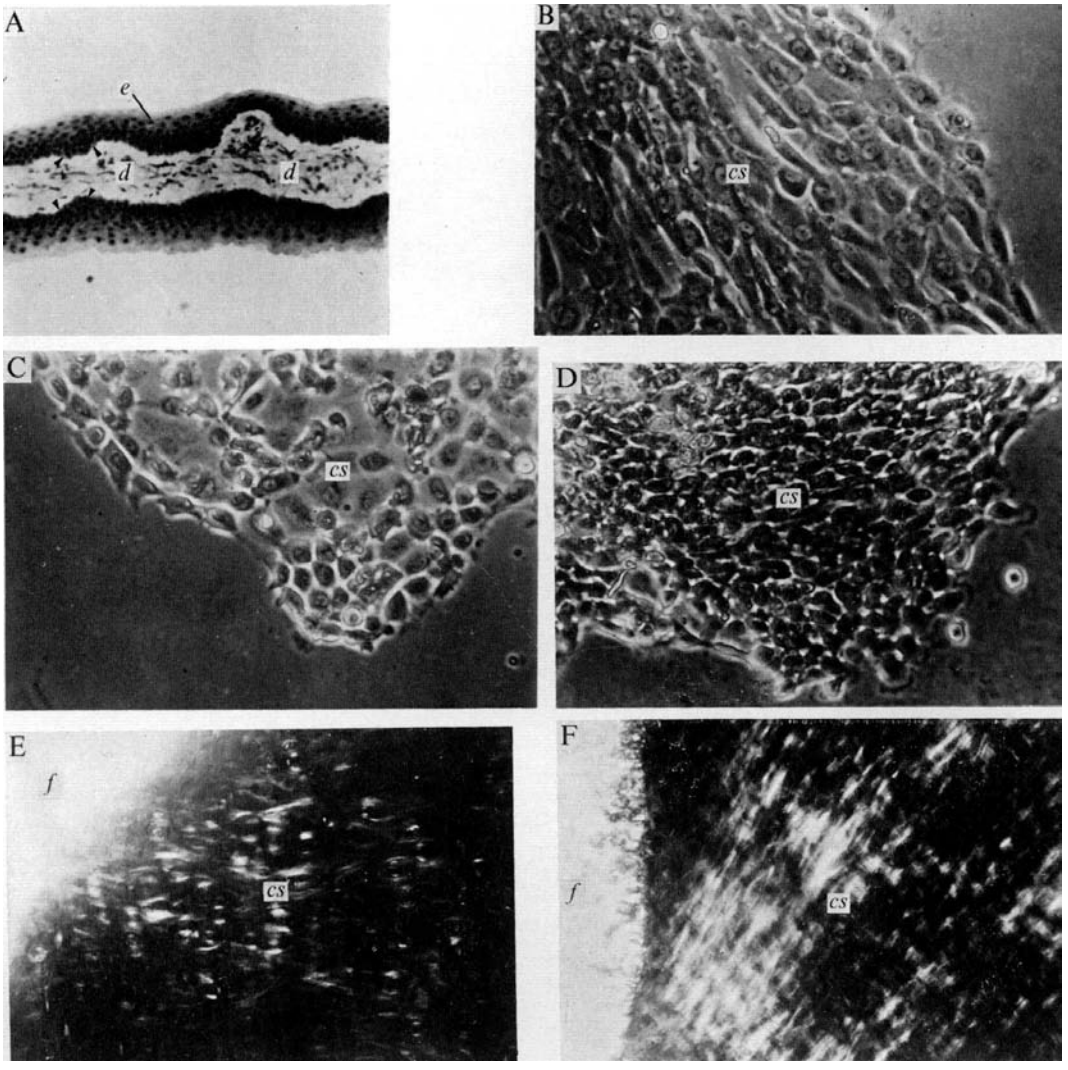

Fig. 1. Morphology of foot-web skin (A) and monolayer cultures derived from it (B–F).

(A) Transverse section through the foot web of an adult *Xenopus* stained with Harris' haematoxylin and light green. × 100. Epidermis (*e*), primarily a layer of epithelial keratinocytes 4–10 cells in thickness, covers the upper and lower surfaces of the web. The dermis (*d*), a loose connective tissue matrix containing fibroblasts and other cell types, forms the inner core. Between the epidermis and the dermis is a thin, highly convoluted, acellular layer, the basement membrane. The arrows point to the basal cell layer (immediately adjacent to the basement membrane) which is the site of most of the cellular proliferation in the epidermis.

(B), (C), (D) Phase-contrast photographs of the leading edge of a living epithelial cell sheet (*cs*) that has migrated out on to a collagen substrate from a small explant of adult web cultured in DME (see Methods section). Morphologically this monolayer of cells is almost completely epithelioid in character with little contamination by other cell types such as fibroblasts or melanocytes. This series of photographs shows the progressive keratinization of the cells within the sheet as a function of time in culture. (B) a 3-day culture; (C) a 6-day culture; (D) an 8-day culture. × 230.

(E, F) Polarized light photographs of living cell sheets showing the increasing cytoplasmic birefringence that occurs with increasing keratinization of the cells *in vitro*. The explants were grown directly on glass coverslips without collagen. The explanted fragment (*f*) is extremely birefringent and the adjacent cell sheet (*cs*) increases in birefringence with differentiation. The medium is isotropic and the cornifying cells become increasingly anisotropic as they fill with coherent, parallel, birefringent fibrils. (E) A 3-day culture. × 250. (F) A 6-day culture. × 280.

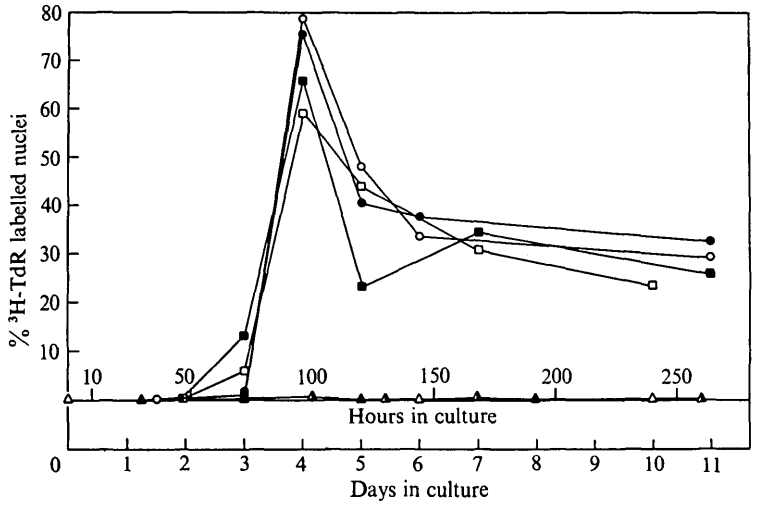

Fig. 2. Comparison of the relative numbers of cells engaged in DNA synthesis in the cellular outgrowths from various tissue explants grown on collagen substrate as a function of culture time. Long pulses (12 h) of [^{3}H]thymidine (10 μCi/ml) were given to cultures on successive days. The cultures were fixed, the explant fragments removed, and the cell sheets remaining attached to the coverslips were prepared for autoradiography. After exposure and development of the emulsion, the percentage of cells with labeled nuclei was determined by a count of at least 1000 cells. Each point is the average of three different cultures. △——△, Web cultures in DME; ▲——▲, web cultures in L15; ○——○, heart cultures in DME; ●——●, heart cultures in L15; □——□, lung cultures in DME; ■——■, lung cultures in L15. Note: the web cultures do not incorporate appreciable amounts of [^{3}H]thymidine at any time during the culture period.

Fig. 2 shows that the cell sheets which migrate out from adult skin explants cultured on collagen differ from other cell types in that they fail to incorporate [^{3}H]thymidine. When lung or heart explants are cultured on collagen and exposed to long pulses (12 h) of [^{3}H]thymidine (10 μCi/ml) on successive days, a synchronous wave of DNA synthesis is observed between 3 and 4 days in culture (Fig. 2). The populations derived from these sources then continue to synthesize DNA asynchronously for prolonged periods. In contrast, no appreciable thymidine incorporation is detectable in the monolayer sheets of cultured adult skin cells treated in an identical way (Fig. 2). Of over 10000 epithelial cells scored autoradiographically only six had incorporated low levels of [^{3}H]thymidine. Furthermore, when cultures were exposed continuously to [^{3}H]thymidine (for up to 6 days), only 5 out of 8000 cells had incorporated any detectable label (and even these cells had very few autoradiographic grains over their nuclei). Likewise, monolayer skin cultures on glass or collagen exposed to colchicine (10^{-4} M) continuously for periods of up to 5 days do not accumulate any mitoses. Identical results were obtained with both culture media (DME and L-15). The possibility that these cells are impermeable to both thymidine and colchicine is highly unlikely since other precursors including

[^{3}H]uridine and amino acids are taken up and incorporated efficiently. Furthermore, in other cell types mitoses can be observed without the use of colchicine. Therefore we conclude that the terminal events of differentiation of these skin cells in culture occur without cell division.

The synthesis of keratin-like proteins

Another indirect indication of true epidermal cytodifferentiation, in addition to the above mentioned increase in cytoplasmic birefringence, is the progressive increase in capacity of the cells to take up histochemical stains that selectively stain for protein thiol or disulfide groups (stains such as mercury orange and performic acid–alcian blue). Since the epidermis of *Xenopus* is known to contain keratin-like proteins with a relatively high cysteine content (Reeves, 1975) both the staining and polarization results are consistent with the above observations being expressions of *in vitro* differentiation.

To monitor the rates of cysteine incorporation in the differentiating skin cultures, [^{3}H]cysteine (20 μCi/ml) was added for 2 h pulse periods on successive days of culture. The amount of radioactivity in acid-insoluble material (cold 5 % TCA) was determined by scintillation counting. In normally differentiating cell sheets the rate of cysteine incorporation remains relatively constant, with a slight increase in incorporation on about day 5 until about day 6 when it drops very rapidly as the cells completely keratinize.

However, to adequately demonstrate a true cellular differentiation it is necessary to be able to show the elaboration of a tissue specific, biochemically defined product by the cells involved. In a previous report (Reeves, 1975), two distinctive keratin-like proteins (labeled bands I and II on polyacrylamide gels) were characterized biochemically. These proteins were found only in the epidermis of post-metamorphic frogs. If true keratinization is occurring within the cultures of adult epidermal cell sheets it should be possible to demonstrate either synthesis or accumulation of such proteins in the cultured cells. Fig. 3 shows that indeed these epithelial outgrowths are synthesizing keratin-like proteins (in addition to some other proteins not seen before) that are characteristic of *Xenopus* epidermis. Cultures were labeled with medium containing 20 μCi/ml of [^{3}H]cysteine for 24 h and then keratin-like proteins extracted from the monolayer and separated by electrophoresis on poly-acrylamide gels as described in the Methods section. From Fig. 3 it is evident that the major fraction of the incorporated radioactivity is found in proteins that co-migrate with marker KLPs extracted from adult skin epidermis and electrophoresed on a parallel gel (bottom of figure). Between 80 and 85 % of the incorporated [^{3}H]cysteine is recovered as radioactivity in the SCM-protein fraction of the total epidermal proteins. In Fig. 3 there also appear to be a number of smaller radioactive proteins, or perhaps incomplete peptides, present as faster migrating species on these gels. However, the bulk of the incorporated cysteine co-electrophoreses with KLP bands I and II.

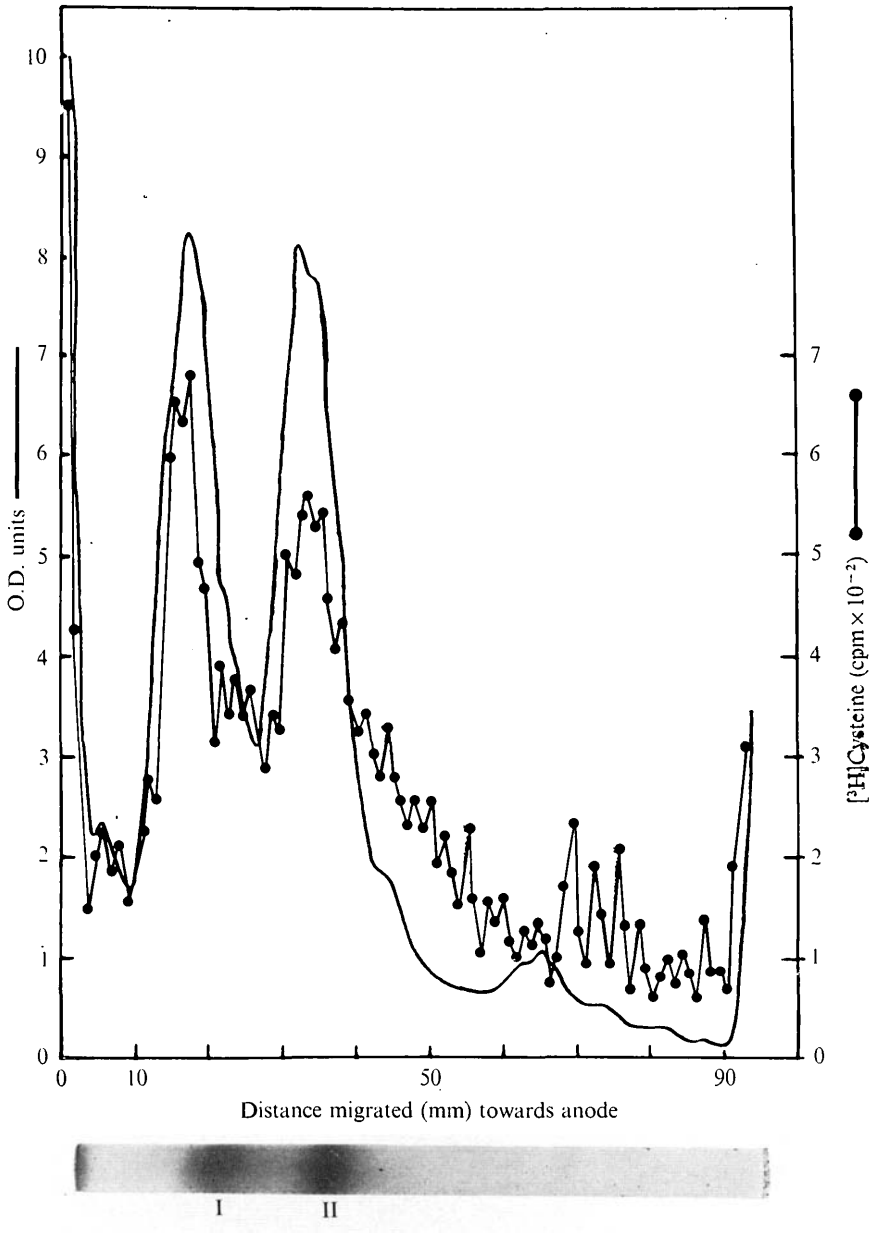

Fig. 3. Incorporation of ^{3}[H]cysteine into the keratin-like proteins of web epithelial cell sheets cultured for a total of 4 days. Polyacrylamide gel electrophoresis was used to separate keratin-like proteins extracted from four cultures labeled with ^{3}[H]cysteine (20 μCi/ml of DME) for 24 h, starting from day 3 of culture. The medium was removed, the cultures washed with Barth X, and the web fragments completely dissected away leaving the cell sheets attached to the dish. These sheets were scraped off with a rubber policeman and combined with 10 mg of unlabeled 'carrier' epidermis isolated from the skin of an adult frog. Keratin-like proteins were extracted from the combined tissues as described in Methods. Sixty μg of the protein extract was electrophoresed on two separate disc gels. One gel was stained for proteins with Coomassie brilliant blue (the bottom of figure) and the optical densities of the stained bands were determined with a Gilford Scanning Spectrophotometer. A parallel gel was sliced and counted for radioactivity (upper graph).

We conclude so far that the epithelial monolayers have the following characteristics: (1) they appear to be a morphologically homogeneous population of epidermal cells; (2) they appear to undergo progressive and near synchronous differentiation *in vitro* without undergoing simultaneous DNA synthesis or cell division; and (3) they synthesize proteins that preferentially incorporate cysteine and have electrophoretic mobilities similar to those of true epidermal KLPS.

The results reported so far do not give a quantitative estimate of the proportion of cells within the population that are actually synthesizing keratin. To answer this question the cell sheets were reacted with fluorescein-labeled anti-KLP antibody preparations that were monospecific for band I type of epidermal proteins. The results of such an antibody reaction are shown in the fluoromicroscopic photograph of Fig. 4. It is seen that all of the cells within this 3-day-old epidermal monolayer cultured on a collagen substrate have reacted with the anti-keratin antibody and show generalized fluorescence. The brightly fluorescing, rounded cells are completely keratinized dying cells. Rounded or dying cells from other tissues do not bind this antibody. Less than 1 in 2000 skin cells grown on a collagen substrate fails to react with the fluorescent antibody preparation. Out of a total of 6700 epidermal monolayer cells observed under phase contrast microscopy and then scanned under ultraviolet light to score individual cells for fluorescence, only three cells within the sheets failed to react with the antibodies and thus lacked detectable cytoplasmic fluorescence. These non-fluorescing cells were generally of fibroblastic morphology, were usually found near the periphery of the outgrowths, and appeared to have migrated out from the web fragments ahead of the coherent sheets of cells.

Just before a cell undergoes complete terminal keratinization it sometimes shows what appears to be nuclear fluorescence when reacted with fluorescein-labeled antibodies. Some, but by no means a majority, of the cells within a population show such fluorescence. Whether this nuclear reactivity toward antibody is indicative of an accumulation of KLPs within the terminally degenerating nucleus as suggested by some (cf. Montagna & Lobitz, 1964) or merely represents keratin-like proteins accumulating in the cytoplasm over the nucleus is not known at the present time.

From the earliest time that skin monolayer outgrowths are large enough to be dissected away from the explant for assay with fluorescent antibody (after 1–1½ days of culture) virtually all of the cells show cytoplasmic fluorescence which increases in intensity with time in culture as the cells become increasingly differentiated.

This fluorescein-labeled antibody preparation does not react with control cultures set up from adult lung, kidney, brain or heart. Likewise, it failed to react with a permanent line of cultured embryonic *Xenopus* cells. The fluorescence labeling is thus specific for adult epidermal cells undergoing keratinization. It is therefore concluded that the epithelial monolayer that migrates

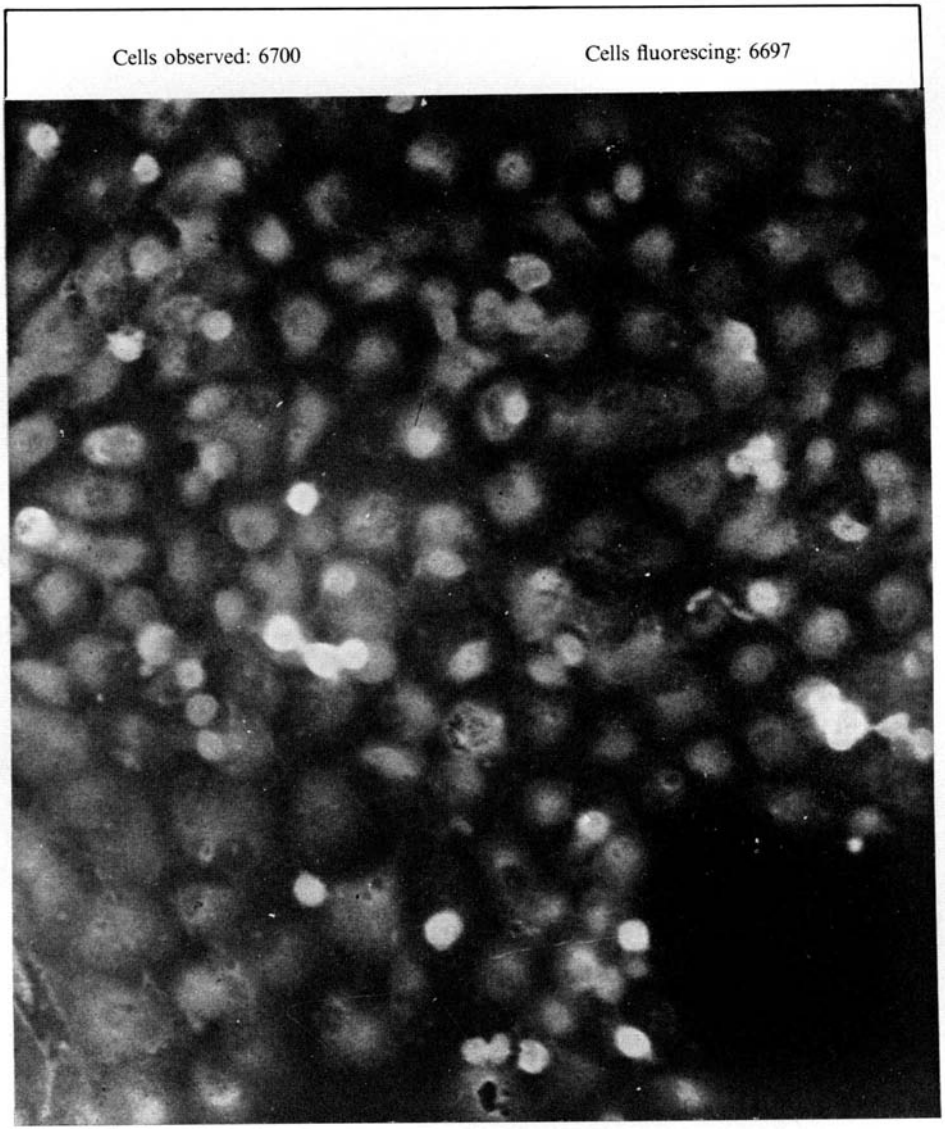

Fig. 4. Binding of fluorescein-labeled monospecific anti-keratin antibody to an acetone fixed epithelial cell sheet cultured for 3 days on collagen as described in the text. After reaction with antibody the cell sheet was photographed with ultraviolet light. Almost all of the cells that have migrated out of the explants on to the substratum are seen to have a rather uniform cytoplasmic fluorescence. The brightly fluorescing rounded cells are dead, completely keratinized cells that have detached from the cell sheet. Monolayer cultures from non-keratinizing control tissues such as heart, lung and brain do not react with the antibody preparation. × 500.

out from web explants cultured on a collagen substrate is essentially a homogeneous array of cells with the same biochemical phenotype.

Environmental modulations of differentiation

The epidermis has long been regarded as one of the most developmentally labile of all differentiating vertebrate tissues and is very sensitive to phenotypic modification by environmental factors (Montagna & Lobitz, 1964). During embryonic development the most important external factor influencing the differentiation of the epidermis is the type of subjacent dermis present (Fleishmajer & Billingham, 1968) or the type of substratum that the epidermis is grown on if cultured *in vitro* (McLoughlin, 1961*a*, *b*; Dodson, 1967*a*). However, one of the most thoroughly investigated of the natural environmental modulations of epidermal cellular phenotype is the effect of vitamin A on differentiation (Fell & Mellanby, 1953; Fell, 1957; Lawrence & Bern, 1963; Beckingham Smith, 1973*a*, *b*, *c*). A deficiency of this vitamin causes secretory epithelia to lose their glandular structure and function and to undergo a squamous metaplasia leading to keratinization (see review by Wolbach, 1953). Vitamin A excess, on the other hand, completely inhibits normal keratinization of the epidermis and instead leads to mucous metaplasia, transforming the epidermis into an actively secreting tissue (Fell & Mellanby, 1953).

This vitamin-induced modulation of epidermal cell phenotype between the keratinizing and secretory states has been postulated to be the result of various concentrations of the vitamin acting directly on the dividing generative basal cells causing these cells to subsequently follow either one or the other of these courses of differentiation (Fell & Mellanby, 1953; Fell, 1957). That there may, however, be more flexibility within these differentiated cellular phenotypes than this 'either/or' situation suggests, is shown by the finding that in some embryonic chick skin cells recovering from hypervitaminosis A both mucin and keratin are produced within the same cell (Fitton-Jackson & Fell, 1963). Likewise, recent electronmicroscopic studies of normally differentiating epidermis of the frog *Rana pipiens* have revealed both mucin droplets and keratin fibers existing within the same differentiating cells (Lavker, 1974).

All previous reports of environmentally induced modulations of epidermal cell phenotype have involved studying heterogeneous populations of both dividing and non-dividing cells (Fell, 1957; Pelc & Fell, 1960; Dodson, 1967*a*, *b*). It has thus been difficult to experimentally distinguish between the effects of such environmental factors on the dividing basal cells relative to their direct effects on the non-dividing cell populations present that have already started to follow a certain course of cytodifferentiation. It was thus of interest to investigate whether environmental factors could directly influence the differentiation fate of the homogeneous, non-dividing epidermal cell populations reported on here. Two types of study were therefore undertaken. In the first type of experiment excess vitamin A was added to the cultures to see if overt

mucous production could be induced in non-mitotic epidermal cells. In the second, an antagonist of vitamin A, citral alcohol (Aydelotte, 1963*a*, *b*) was added to the cultures to investigate whether the rate of cellular differentiation, namely keratinization, is an intrinsic feature of the cellular 'program for differentiation' or is amenable to influence by environmental factors.

Cultured skin explants generally lose their epithelial outgrowths when keratinization is completed. Thus the percentage of explants retaining their monolayer outgrowth, as a function of time in culture, is an indirect measure of the rate of keratinization (Fig. 5A). A more direct measure is obtained by reacting the carboxymethylated proteins extracted from the epithelial sheets with ^{125}I-labeled monospecific antibody prepared against one type of skin keratin (Fig. 5B). Carboxymethylated extracts from a non-keratinizing permanent *Xenopus* embryonic tissue culture cell line were used to determine the background level in this assay. Comparison of Figs. 5(A) and (B) shows that as synthesis of immuno-reactive keratin by skin cells progresses, so the viability and adherence of the monolayer to the substratum decreases. Vitamin A and citral each affect both parameters. In cultures treated with excess vitamin A there is an exceptionally slow accumulation of immuno-reactive keratin (Fig. 5B) and concomitantly, the average monolayer retention time of explants is increased (Fig. 5A). Conversely, treatment of the cultures with citral greatly accelerates both the synthesis of immuno-reactive keratin (Fig. 5B) and the rate of loss of monolayer cell sheets from the explants (Fig. 5A).

Neither citral nor vitamin A changes the direction, but only the rate, of differentiation of these skin cells. Thus, although the epithelial cell sheets normally have a low, but finite amount of mucous-like material in them as shown by light cytoplasmic staining with PAS or other stains for mucopolysaccharides (Pearse, 1968), if excess vitamin A is added to the cultures there is no detectable increase in mucous production within these non-mitotic cells. Furthermore, citral does not change the total amount of keratin accumulated in the lifetime of the cells, only the rate of its accumulation. In both untreated and citral-treated cultures the amount of immuno-reactive keratin accumulated by these cells *in vitro* is similar to the level in adult epidermis which has differentiated *in vivo* (Reeves, 1975). It is noteworthy, however, that this *in vitro* cornification appears to be a much slower process than naturally occurring, *in vivo*, amphibian skin keratinization (Lavker, 1974).

DISCUSSION

The change or sequence of changes which eventually lead a cell to undergo cytodifferentiation are often considered to be 'internally programmed'. That is, once the program for a particular type of differentiation has been set, the cell's ability to eventually express this program seems to be very stable (Ursprung, 1968) and relatively independent of external environmental influences. The

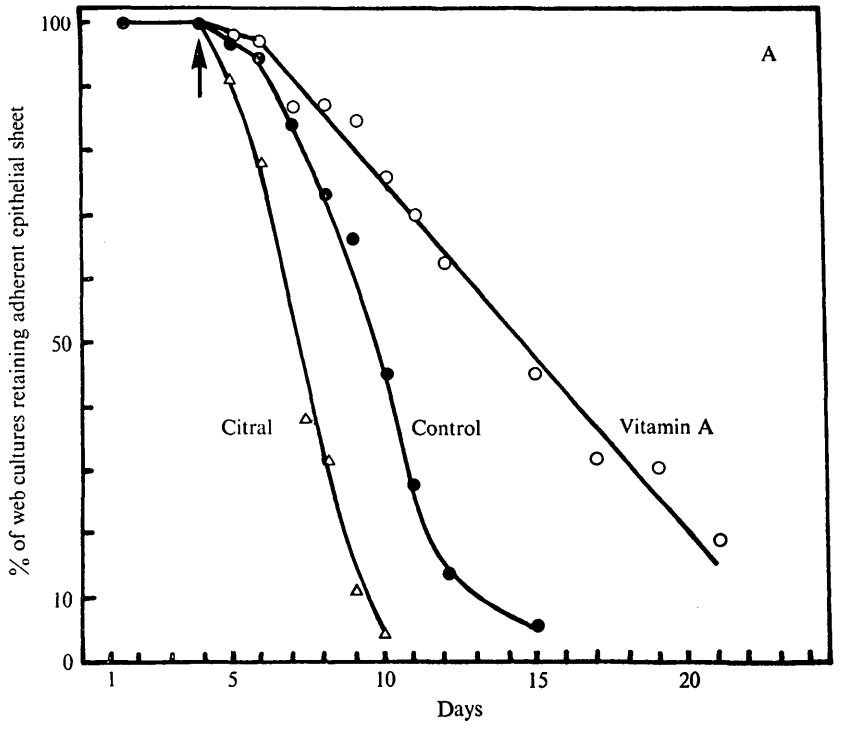

Fig. 5. Influence of vitamin A and citral on the rate of *in vitro* keratinization of monolayer cell sheets.

(A) Effect on cell survival. As described in text, the rate of loss of epithelial outgrowths from cultured explants is an indirect measure of the rate of keratinization. All cultures were grown in DME on collagen. Each point is the average of three cultures. ●——●, Normal web cultures; ○——○, excess vitamin A (25 i.u./ml) added on day 4 (arrow); △——△, citral (0·1 %) added on day 4.

(B) Effect on accumulation of proteins which react with anti-(band I) keratin antibodies labeled with I^{125}. See text for details. Culture conditions and figure legends as in Fig. 5A except that vitamin A and citral were added to the experimental cultures on the third day of culture (arrow). Each point is the average of four cultures with the S.E.M. indicated by error bars. The amounts of band I type keratin-like proteins within the SCM-protein extracts (right-hand axis) were determined by referring the amount of radioactivity in the experimental antigen/antibody complexes to a standard curve produced by reacting the same antibody preparation (with a specific activity of $1{\cdot}95 \times 10^5$ cpm/μg protein) with known amounts of electrophoretically purified band I KLP incorporated into agar as described in Methods.

In this series of experiments the SCM-proteins, as a fraction of the total epidermal proteins, averaged 52·2 ± 6 %.

epithelial cells described in this report support this concept. The skin cells that migrate out of an explant on to a collagen or glass substrate have obviously been programmed to undergo terminal keratinization and will eventually follow this course given appropriate culture conditions. However, as shown in Fig. 5, this differentiation is not entirely independent of external influences

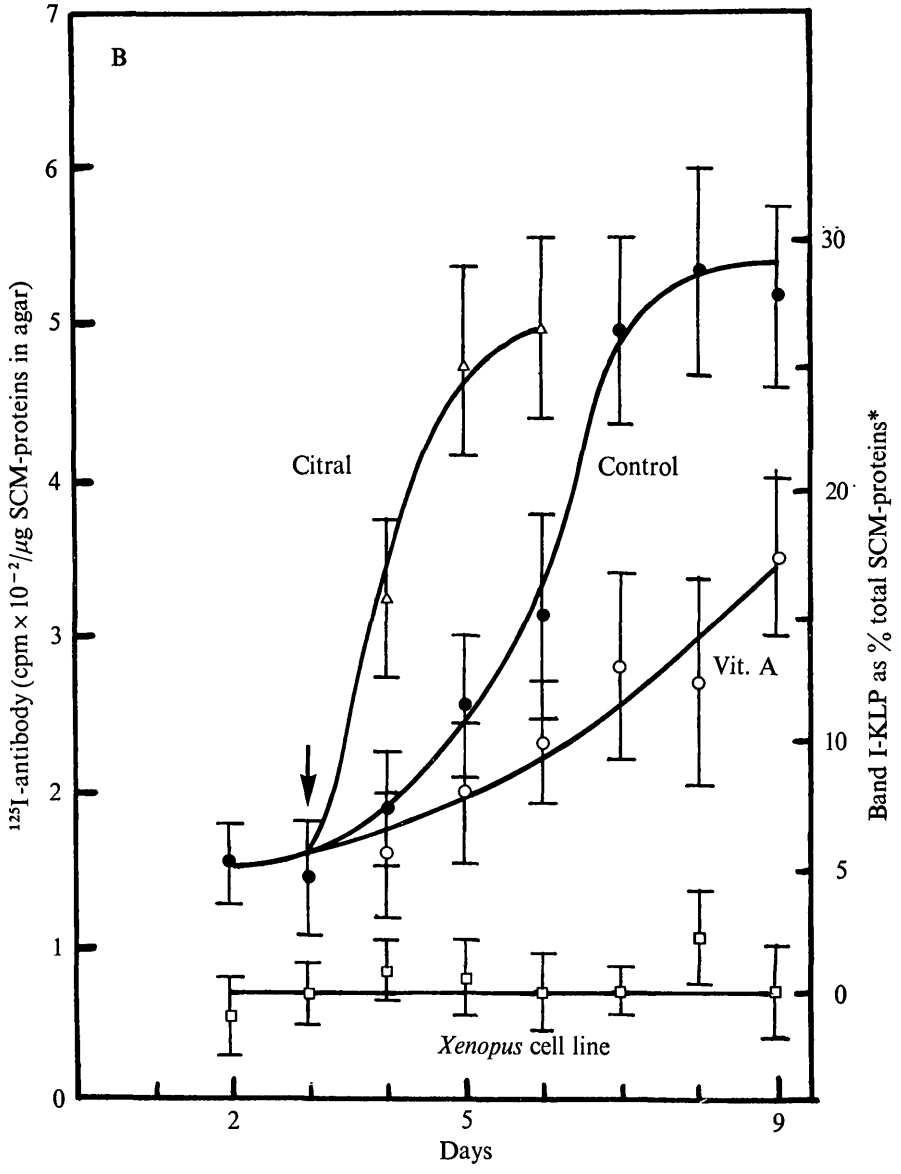

Fig. 5B. For legend see opposite.

since excess vitamin A added to the culture medium can slow the rate of keratinization considerably whereas the addition of citral can greatly accelerate the rate of cornification.

Thus, in common with other systems in which keratinization has been studied (Fell & Mellanby, 1953; Fell, 1957; Lawrence & Bern, 1963; Dodson, 1967*b*; Aydelotte, 1963*a*, *b*; Beckingham Smith, 1973*a*, *b*, *c*), the homogeneous population of keratinizing cells which migrates out from cultured frog skin

responds to vitamin A and citral. However, in this non-mitotic cell population, these factors appear to change only the rate of differentiation, not its direction.

The role that cell division plays in the programming or re-programming of cells for certain types of differentiation has been the focus of much experimentation and speculation. Although the non-dividing skin cells did have a low, but detectable, amount of material with the histochemical staining properties of mucopolysaccharides, no equivalent of overt, copious mucous production (Fell & Mellanby, 1953) in the presence of excess vitamin A was detected. In all previous reports where vitamin A has been used to induce mucous metaplasia in keratinizing epithelia, when the responding tissues were analyzed it was found that there was a complex mixture of populations of dividing and non-mitotic cells (Fell and Mellanby, 1953; Fell, 1957; Pelc & Fell, 1960; Lawrence & Bern, 1963; Dodson, 1967*a*, *b*). The results reported here for cultured amphibian epidermal cells exposed to vitamin A are thus compatible with the suggestion, first made by Fell (1953, 1957) that DNA synthesis and cell division are necessary prerequisites for the induction of epithelial mucous metaplasia. However, other explanations for the current findings, such as the age of the epithelial tissue used, the species source of the tissue, or the lack of sensitivity of the histochemical staining techniques to detect low levels of mucous production, cannot be ruled out except by further experimentation.

All of the evidence in the present report indicates that the monolayer of epithelial cells that first migrates out from a cultured web explant onto a collagen or glass substrate is a biochemically homogeneous population of cells that has been committed to undergo terminal keratinization. These cells, before they completely cornify, can be considered to be in a 'protodifferentiated' state (Rutter *et al.* 1968) or perhaps more appropriately, in an early state of phase II differentiation as defined by Kafatos (1972). This state is characterized by the synthesis of low levels of differentiated product by cells which have undergone a 'quantal division' (cf. Holtzer, Weintraub, Mayne & Mochan, 1972) and are committed to differentiate in only one direction, in this case to keratinize. This should be a useful culture system for the study of the biochemistry of terminal cell differentiation.

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