*SUBSTRATE AND NUTRIENT EFFECTS UPON EPIDERMAL BASAL CELL ORIENTATION AND PROLIFERATION**

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Amniote epidermis is a favorable tissue for study of the relationship between cytodifferentiation and cell division. A germinal population is maintained in the epidermal basal region, and daughter cells passing outward through the epithelium synthesize keratins, lose nuclei, and become cornified. At different times, and in different parts of the embryo, proliferating basal cells are characterized by varying degrees of columnarity or palisading, and by varying mitotic activities. Both of these features are influenced by the type of mesenchymal cell in proximity to the basal layer.[1, 2] The possibility of assaying factors that affect these parameters of epidermal behavior was suggested by Dodson's preliminary report.[3]

The present work demonstrates that epidermal basal cells can maintain columnar orientation, thymidine-H^3 incorporation, and mitosis in the absence of living dermal cells, providing that a suitable physical substrate is available to basal cells and that a macromolecular fraction of chick embryo juice is present as nutrient. This suggests that the epidermal response is evoked by products of mesenchymal cells even under conditions where nonliving substrates appear to support epidermal activity.[3]

Methods.—Skin tissues were taken from the anterior tarso-metatarsal region of 11-day (stage 37) chicken embryos. Tissue culture methods, composition of the protein-free nutrient medium (a variation on Weymouth's MB 752/1), labeling procedures, and histological-autoradiographic techniques were as described earlier.[1, 2]

Epidermis was separated from dermis by treatment with trypsin (2.25%) and pancreatin (0.75%) prepared in Ca-Mg-free Tyrode's solution and used at 3–6°C.[4] Epidermis was freed as an intact sheet after 10–15 min of treatment. Virtually no adhering dermal cells remained affixed to the lower epidermis and all visualizable capacity to stain with periodic acid-Schiff reagents was lost.[1] Other pieces of skin were placed in 0.04% disodium ethylene diamine-tetra-acetate[3] prepared in Ca-Mg-free Tyrode's, and used at 3–6°C; the epidermis was freed as a sheet after *ca.* 20–25 min. No differences were exhibited by epidermis separated by these two methods, and both groups are treated together in the *Results* section.

Tropocollagen (from calfskin; a gift of Dr. Jerome Gross) at 2 mg/ml in Na_2HPO_4 buffer, pH 7.6, ionic strength 0.4, was used in making gels. Several small drops of the chilled solution were placed on each side of Millipore filter platforms that had been prewet with protein-free nutrient medium. In some cases 3:1 mixtures of tropocollagen and medium were placed on the filter. The assembly was then incubated under moist-chamber conditions at 37°C, 5% CO_2, for at least 3, and usually 24 hr. For use, the assembly-filter and firm collagen gel were immersed in nutrient medium, and the epidermis to be tested was placed over the gel, basement membrane-side down. The assembly was then transferred to culture conditions.

Freezing was used to kill dermal cells.[3] In some cases, an assembly surmounted by mesenchyme was frozen solid at a temperature of −2 to −11°C in 15–30 sec. It was thawed in nutrient medium at 3–10°C. Alternatively, several pieces of dermis suspended in a small volume of Tyrode's solution in a sealed test tube were frozen in an ethyl alcohol-water-dry ice mixture at −25°C. Following three successive freeze-thaw cycles, the pieces of dead dermis were placed on filter platforms, and freshly separated living epidermis was placed in direct contact with them.

Embryo juice (EJ) was prepared from groups of 3–4 dozen 9-day embryos and was fractionated according to the method of Rutter[5] as follows: (1) eyes removed and discarded; (2) embryos expressed through stainless steel grid in 50-ml syringe; (3) equal volume of ice-cold Tyrode's added; (4) incubated at room temperature 30 min; (5) centrifuged (3°C) at 1350 *g*, 10 min;

(6) supernatant removed and frozen with dry ice; (7) thawed in 37°C waterbath (this fluid is "whole embryo juice" and is stored frozen); (8) centrifuged (3°C) 2000 *g*, 10 min; (9) loose 2000-*g* pellet suspended in cold Tyrode's (one quarter the volume submitted to step 8); (10) 2000-*g* supernatant centrifuged in a Spinco Model L, 0°C, 60 min, 105,000 *g*; (11) upper white supernatant layer and clear yellow supernatant removed, frozen separately; (12) firm pellets from 2–4 tubes suspended in cold Tyrode's (total volume one quarter that of volume submitted to step 10—these pellets either were broken up by violent stirring in a chilled tube, or by hand homogenization using a chilled Potter-Elvehjem homogenizer. All fractions were frozen in small aliquots and were used once immediately after being thawed (fractions tested within 3 days of preparation).

Fractions submitted to heating were placed in small test tubes immersed in water at 37°, 60°C, or boiling for 15–20 min. A flocculent precipitate resulting from treatment at the latter two temperatures was removed by centrifugation at 1350 *g* for 10 min.

Dialysis of embryo juice or fractions was carried out in standard dialysis tubing, against several liters of Tyrode's solution at 2–3°C for 18–24 hr (stirred constantly).

Whole embryo juice and 100,000 *g* fractions were treated prior to use with crystalline trypsin (California Biochemical Corp.; 0.1 and 1.0 mg/ml in Tyrode's; 37°C, 1 or 2.5 hr), ribonuclease (Worthington; 0.1 mg/ml, 37°C, 1 hr), and deoxyribonuclease (Worthington; 0.1 mg/ml, 37°C, 1 hr). Soybean trypsin inhibitor (0.1 and 1.0 mg./ml) was added to the trypsinized fractions. Trypsin inhibitor and trypsin were added to a control tube of the fraction under test. Other control tubes were incubated in the same manner as experimental tubes. As an alternative procedure, a 100,000-*g* pellet-homogenate was incubated at 37°C for 2 hr with trypsin at 1.0 mg/ml. Thereafter, centrifugation at 100,000 *g* for 1 hr sedimented a soft pellet which was homogenized and tested. Following these treatments, samples were used at the 20% level with defined medium.

The 100,000-*g* pellet (homogenized in Tyrode's) obtained from 1- or 8-hr centrifugation was treated with sodium deoxycholate, 0.65 or 1.0%. The mixture was layered on top of an equal volume of 0.3 *M* sucrose in Tyrode's and was centrifuged at 100,000 *g* for 1 hr. After discarding the supernatant layers, the pellet was washed repeatedly with Tyrode's, and was suspended in Tyrode's by homogenization or stirring. Controls included an aliquot of the original pellet homogenate, and an aliquot run through the sucrose centrifugation procedure but not treated with deoxycholate. Alternatively, the deoxycholate-homogenate mixture was centrifuged at 100,000 *g* for 1 hr, and then the pellet was handled as above, whereas the supernatant was dialyzed by the usual procedure prior to test.

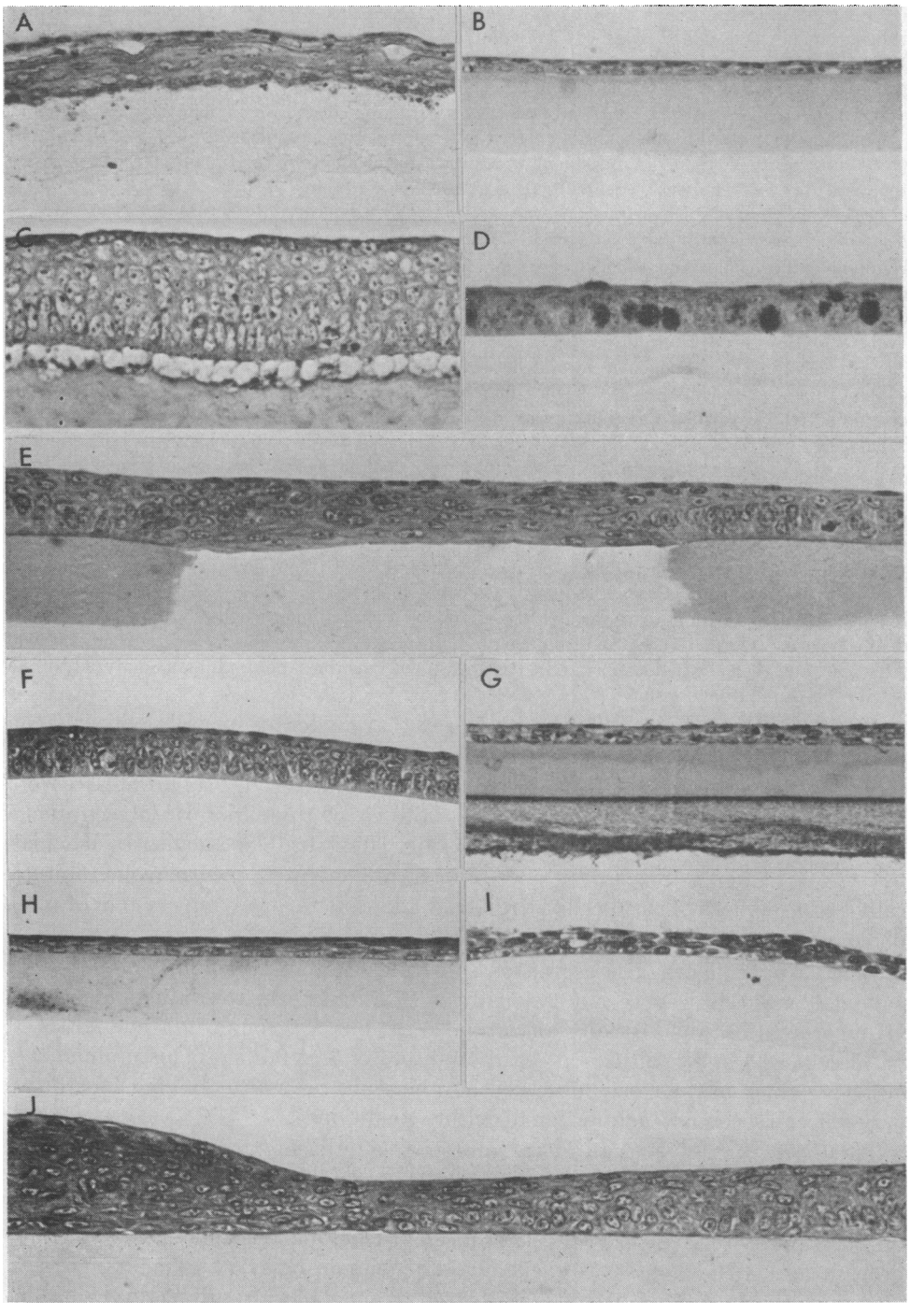
Results.—Epidermis combined with frozen-thawed dermis or with tropocollagen gels fails to retain columnar, oriented basal cells, or proliferative activity when cultured in defined nutrients. In 35 recombinants in which all dermal fibroblasts were killed, no columnar epidermal basal cells, mitoses, or thymidine-H^3-labeled nuclei were detected after 24, 48, or 96 hr *in vitro* (Fig. 1*A*). In 13 of 24 cultures, in which the dermis had been frozen only once prior to use, oriented, incorporating, mitotically active epidermal basal cells were found adjacent to surviving clusters of morphologically normal mesenchyme cells.

When tropocollagen gels served as substrate to the epithelium (32 cultures), cuboidal basal cells were seen occasionally at 24 hr, but never at 48 hr. Thymidine-H^3 incorporation was virtually absent at 24 hr, but was found in a few flattened basal cells in 4 of 13 cultures at 48 hr, and in 5 of 8 at 96 hr. This proliferative activity, which has not been observed in other cultures with nonliving substrates, may reflect a secondary adaptation to culture conditions.

These observations on cultures in protein-free nutrients are strikingly different from those of Dodson[3] who cultured similar recombinants on chicken plasma-embryo juice clots. Therefore, recombinants were run on clots made of a 1:1 mixture of fresh adult cock plasma and 9-day chick embryo juice (see above for preparation). In 18 of 25 cases, cuboidal or columnar basal cells were found when epidermis was in contact with frozen-thawed dermis. As a result of this confirma-

tion of Dodson, plasma, embryo juice, horse serum, and other large-molecular substances were tested in combination with the defined nutrients.

In brief, horse serum (10 or 20% by volume, 6 explants), the Cohen epidermis



stimulating protein[6] (O.D. 280 mμ 0.01, 8 explants), tropocollagen (2–40 μg/ml, 9 explants), bovine serum albumin[7] (California Biochemical Corp., cryst.; 1 mg/ml, 4 explants), and chicken plasma (20%) all failed to duplicate the effect of a complex clot. Oriented basal cells were absent from all cultures, except 3 of 7 grown with plasma present; there some cuboidal basal cells persisted until 48 hr *in vitro*.

Completely different behavior resulted when embryo juice (20% by volume) was added to the defined nutrients. Large areas of columnar basal cells, with normal mitosis and thymidine-H^3 incorporation, persisted. Embryo juice produced these effects when epidermis was combined with frozen-thawed dermis (15 cultures), tropocollagen gels (7 cultures) (Fig. 1*C*), and Millipore filters (79 cultures) (Fig. 1*B*, *D*), but not when the epithelium rested upon lens paper (11 cultures).

The only obvious difference between lens paper and Millipore filter is the amount of surface available as substrate to individual tissue cells. Most cells fail to contact the lens-paper fibers, and therefore are exposed directly to the fluid medium. A similar situation was achieved by punching 4 holes of 0.15-mm diameter each in Millipore filters prior to adding epidermis. In control cultures in defined nutrients, basal cells on the filter and over the holes were equally flattened. In all of 28 cultures with 20 per cent embryo juice, cells above the filter were oriented and mitotically active, whereas those above the hole were quite flat (Fig. 1*E*). The transition point from columnar to flattened occurred directly at the edge of the hole. Thymidine-H^3 incorporation was drastically reduced in frequency above the hole also, again indicating that the oriented condition and proliferative activity are closely linked properties of the basal germinal population.[1, 2]

Difference in behavior of epidermis over filter as opposed to over hole could be due to the fact that spreading or flattening substances might be unable to pass the filter matrix. To test this possibility, whole embryo juice was passed through the same type Millipore filter used for culturing (*ca.* 25 μ thick; pore diameter 0.45 μ; held in Swinny adapter at 37°C). In another experiment, epidermis on a filter (HA type; 150 μ thick) with holes was placed directly over an intact filter (TH type; 25 μ thick), so that all nutrients had to pass through filter matrix prior to reaching the epithelium. Results from both series were identical in showing an absence of orientation over holes and a normal germinal layer over filter.

Another substrate tested for ability to support orientation was the pellet obtained from centrifugation of EJ between 2000 and 100,000 *g* (see below). Pieces of the

FIG. 1.—(*A*) Epidermis + frozen-thawed dermis; defined nutrients. Basal cells are flattened and little thymidine-H^3 incorporation or mitosis is evident. Autoradiogram. 350×. (*B*) Epidermis on filter; defined nutrients. 350×. (*C*) Epidermis + tropocollagen gel; 20% embryo juice. Note the oriented cells and fibrous processes which attach tissue and gel. Such fibers form in the absence of mesenchymal cells. Phase contrast. 512×. (*D*) Epidermis on filter; defined nutrients + 20% embryo juice. Autoradiogram. 350×. (*E*) Epidermis on filter with hole; 20% embryo juice. Frequent thymidine-H^3 incorporation only occurs in oriented areas. 380×. (*F*) Epidermis on filter; defined nutrients + 20% 100,000-*g* pellet-homogenate. 350×. (*G*) Epidermis on filter which had been cemented above a sheet of cellophane. Although 20% embryo juice was present, the active fraction could not reach the epidermis, and basal cells are flat. (Material below filter is gelatin added during histological processing.) 350×. (*H*) Epidermis on filter; defined nutrients + embryo juice heated near 100°C for 15 min. 350×. (*I*) Epidermis on filter; defined nutrients + homogenate of 100,000-*g* pellet obtained after trypsinization of an original 100,000-*g* pellet-homogenate. 350×. (*J*) Epidermis on filter; defined nutrients + 20% 100,000-*g* supernatant. A transition zone occurs between the oriented cells on right and flattened cells on left. Thymidine-H^3 incorporation occurs only in region of orientation. 350×.

pellet (0.1–0.8 mm^2) upon filters were covered with epidermis so that tissue also contacted the filter around the pellet (24 cultures). If cultured in defined nutrients, all basal cells became flattened; in the presence of 20 per cent EJ, cells contacting the filter were oriented as expected, but those contacting the pellet were flat. The attachment of epidermis to pellet was not strong, since during histological processing most pieces of tissue pulled free of the pellet surface. Therefore, the pellet may be unable to permit normal attachment of basal cells, and, as a result, is an ineffective substrate despite its small "pore" size in relation to individual cells. Alternatively, orientation may be absent because the pellet does not permit passage of the orienting factors of EJ (the homogenized pellet contains such factors; see below).

Control epidermis no longer possesses an oriented, mitotic basal layer after about 18 hr of culture in defined nutrients,[1, 2] but these features appear in response to dermis added at that time. Embryo juice was tested for similar activity. At 24 hr *in vitro*, eight epidermal cultures fixed as controls contained a few scattered patches of cuboidal cells in the predominantly flattened basal layer. Dermis added directly to three and across a Millipore filter to three others evoked establishment of proliferating high-columnar basal cells within 24 hr. In contrast, of 25 cultures treated with 20 per cent embryo juice commencing at 24 hr, 12 contained only flat basal cells when fixed at 48 hr. Scattered groups of cuboidal or high columnar cells were found in the other cultures. Overall, embryo juice seems less effective and consistent than dermis in evoking the epithelial response.

The finding of orientation of basal cells upon filters provides a tool which allows tests of various fractions of embryo juice in the absence of undefined constituents associated with killed dermis or collagen. Sixty-one control explants in defined nutrients and 60 in 20 per cent embryo juice plus defined nutrients were run during the tests. Other controls of epidermis transfilter to dermis or to other epidermis gave, as expected, positive and negative results, respectively. Hence, orientation is dependent upon either embryo juice or mesenchyme transfilter and does not result from covering the opposite side of the filter pores with living epidermal cells.

Whereas in 20 per cent EJ high columnar cells were found in most of the basal region, at 3 per cent EJ (6 cultures) only small patches of cuboidal cells were seen. Infrequent labeled nuclei also testify to the less effective action of the lower concentration. When 50 per cent EJ was used (3 cultures), a normal basal layer persisted, and over-all thickness of the epidermis exceeded that in controls (20% EJ).

Duration of the EJ effect upon epidermis was tested by culturing in 20 per cent EJ-medium for 24 hr, followed by reculture in defined nutrients. When fixed at 48 hr total, all basal cells of four cultures had flattened, and the frequency of labeled nuclei was reduced to that seen in control skin cultured continuously in defined nutrients. Four other controls, in which dermis was removed after 24 hr *in vitro*, also possessed flattened basal regions.

Whole embryo juice remains active following dialysis against Tyrode's solution (13 cultures). A large molecular nature of the active fraction was also suggested by an experiment in which a layer of cellophane was cemented directly beneath each Millipore filter culture platform. All nutrients had to pass both cellophane and filter matrices before reaching the epidermis on top. In four such cultures with 20 per cent EJ present, the basal cells were very flat and were mitotically inactive at 48 hr (Fig. 1*G*). Controls cultured on standard filters or on filters over

cellophane perforated by a 0.2-mm hole showed normal basal orientation and proliferation.

Activity most closely approximating that of whole EJ is associated with the centrifugal pellet which sediments between 2000 and 100,000 *g* (45 cultures) (Fig. 1*F*). Such a pellet was equally active if suspended in ice-cold Tyrode's by vigorous stirring or by homogenization. The 2000-*g* pellet supported only cuboidal orientation and superficially resembled 3 per cent EJ in its effects (11 cultures). Peculiar behavior was noted with the 100,000-*g* supernatant. Presence of either the white upper layer, presumably high in lipids, or the copious clear-yellow supernatant resulted in abnormal thickening of the epidermis that was accompanied by basal cell orientation in scattered areas in 26 of 35 cultures (Fig. 1*J*). The possibility was tested that the small areas of oriented cells might be eliminated by centrifugation of EJ for 8 hr. Aliquots removed from the middle of the supernatant, with great care to prevent mixing of the pellet surface, proved active in that scattered oriented basal areas occurred in all of the eight explants. The residual activity in the 100,000-*g* supernatant also was unaffected by dialysis (6 cultures).

Whole embryo juice and the 100,000-*g* fractions were submitted to various heat treatments. Following incubation at 60°C for 15 min, neither EJ nor fractions supported columnar orientation (25 cultures). In many cultures, however, large numbers of cuboidal basal cells were observed. Even this response was absent when EJ or the 100,000-*g* pellet (homogenized) were incubated in a boiling water-bath for 15 min (8 cultures) (Fig. 1*H*). Therefore, the fraction which supports basal orientation is heat-labile.

Incubation of whole EJ or the 100,000-*g* fractions in trypsin reduced their activity. Cuboidal and flattened cells were seen with EJ incubated in 1.0 mg/ml trypsin, whereas normal high columnar cells occurred after treatment at 0.1 mg/ml. Homogenized 100,000-*g* pellet treated at the higher level supported only questionable cuboidal orientation (9 cultures) (Fig. 1*I*). Interpretation of the results is difficult because most explants failed to attach firmly to the filter (controls in which the treated pellet was mixed with untreated EJ showed attachment; hence presence of inhibitors of attachment does not operate under such control conditions). In contrast to the action of trypsin, ribonuclease and deoxyribonuclease produced no detectable effects upon EJ or the fractions, and basal cells remained oriented even though the enzymes were included in the test medium itself.

The 100,000-*g* pellet homogenate was treated with deoxycholate (DOC) and was resedimented at 100,000 *g*. The homogenate of this second pellet was inactive in supporting orientation in cultures. Similarly, the dialyzed deoxycholate supernatant failed to support orientation of cells attached to the filters. These results are clouded by the presence of necrotic cells and cell debris in the epidermis. Such debris was seen also in control cultures grown in the presence of either DOC-homogenate or DOC-supernatant combined with whole EJ. Therefore, inhibitory substances remain despite efforts to remove them, and the negative results cannot be interpreted.

Discussion.—The experiments reported here confirm that cellular orientation and proliferation in embryonic epidermis are not dependent upon a physical substrate containing living cells.[3] Frozen-thawed dermis, tropocollagen gels, and Millipore filters are equally effective in supporting basal cell orientation under

certain nutrient conditions, whereas lens paper and the 100,000-*g* centrifugal pellet of chick embryo juice are ineffective under all nutrient conditions tested. Spaces between lens-paper fibers are very large in relation to individual cell diameter, while those in the "solid" pellet are small. Hence, no direct argument relating ability to orient with pore diameter of substrate seems tenable (assuming that the pellet is permeable to the active principle of embryo juice). Intimate attachment is seen between epidermis and effective substrates when no orientation occurs (in the absence of embryo juice). Together, these facts argue that the substrate must allow attachment and have some other property perhaps related to the micro-environment at the lower surface of basal cells. Attachment can be thought of as maintaining the cells in an undifferentiated condition.[2, 8, 9] Operation of other factors can then lead to basal cell orientation and proliferation. Differentiation of daughter cells is dependent upon these prior events in the basal population.

Living dermis, chick embryo juice, or certain fractions therefrom, are roughly equivalent in supporting orientation-proliferation *in vitro*. Other substances—horse serum, the Cohen protein,[6] serum albumin,[7] plasma—can affect epidermal behavior or tissue morphology, but none supports maintenance of a germinal layer or development of an epithelium closely resembling that found *in vivo*.

In transfilter culture or in once-frozen dermis that contains surviving clusters of fibroblasts, exceedingly small numbers of mesenchymal cells can support orientation of nearby epidermal cells. Large masses of dermis cultured below a filter lateral to epidermis, or in the bottom of the culture vessel, have no effect upon epidermis on top of a filter (Wessells, unpublished). Relatively large quantities (20% by volume) of embryo juice are required to duplicate the action of a few living cells. Finally, the epidermis seems dependent upon the continuous presence of either living dermis or embryo juice, since removal of either is followed by flattening of the basal cells during the ensuing 10–24 hr.[2] All of these observations imply that a low titer of the active substance(s) is present in embryo juice, and that the substance must be present in continuous supply to maintain normal basal cell activity.

These considerations may be the basis for the only observed difference between embryo juice and living dermis—the reduced ability of the former to evoke orientation-proliferation from preflattened cells. Perhaps different substances or large quantities of the same substances act in that process. Such quantities might never accumulate in the filter matrices when only fluid medium is present. Accumulation may occur when even small numbers of fibroblasts are present, since the producing cells also may effectively seal the lower filter openings and so create a closed micro-environment below the epidermis.

Attempted characterization of the active fraction suggests that it is associated with a particulate component of embryo juice, and that it is trypsin-sensitive and heat-labile (Dodson reports that heat-killed dermis is an ineffective substrate[3]). These characteristics plus the ineffectiveness of the Cohen protein (which is heat-stable[6]) or serum albumin in supporting orientation indicate that the fraction is similar to Rutter's particles[5] that support maturation of the mouse pancreatic epithelium. Although an orientation effect is not easily demonstrated in the pancreas system, stimulation of thymidine-H^3 incorporation and mitosis does occur,[9] just as in chick epidermis.

The locus of action of embryo juice or mesenchyme in either epidermis or pancreatic epithelium is undefined. Operationally, presence of these factors results in compact, nonspreading epithelia with higher mitotic rates than are seen in the spreading explants incubated in their absence. In the case of epidermis, the active factor could be described as antispreading, orientative, or promitotic, but until the mechanism of action is elucidated, none of the terms is particularly useful. Furthermore, since cytodifferentiation of outer epidermal layers or inner pancreatic acini[10] is apparently dependent upon earlier mesenchyme or embryo juice-induced mitosis, it is not clear how the factors relate to first-order differentiative changes in maturing cells. Utilization of nonliving substrates, defined nutrients, and better-defined particulate fractions lends hope for more penetrating analyses of mitosis, tissue mass, and differentiation in the future.

Summary.—Basal cells of embryonic epidermis remain oriented and mitotically active if cultured *in vitro* on a suitable substrate and in the presence of chicken embryo juice or a macromolecular fraction therefrom. Millipore filters, tropocollagen gels, or frozen-thawed dermis are effective substrates, whereas lens paper or 100,000-*g* centrifugal pellets are not. The active fraction from embryo juice is nondialyzable, heat-labile, trypsin-sensitive, and sediments between 2000 and 100,000 *g*.

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*THE ROLE OF D-ALANINE IN THE SEROLOGICAL SPECIFICITY OF GROUP A STREPTOCOCCAL GLYCEROL TEICHOIC ACID**

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Antisera prepared by immunization of rabbits with heat-killed group A streptococcal vaccines often contain precipitating antibodies to a soluble streptococcal antigen which has been identified as polyglycerophosphate.[1] Similar substances reacting with the same antisera are found not only in many different serological groups of streptococci but also in a variety of other Gram-positive species. Purified