Table 1. Titers ($\log_{10}$ $TCID_{50}$*/0.2 ml) of RK-13–adapted rubella virus strains in rabbit-cornea cells.

Virus strain	Cultured cells	
	RK-13	Rabbit cornea
Judith	3.7	3.2
RV	3.3	3.1
Orta	2.5	2.3

* Tissue culture infective dose, 50 percent effective.

larger circular drop, inside which remainders of the nucleus may be seen.

The cytopathic effects in rabbit-cornea cells of the three different strains of RK-13–adapted rubella virus were identical, and titrations simultaneously performed in the two cell lines gave for each of the three rubella virus strains titers of the same level (Table 1).

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Growth of Mouse Mammary Glands in vivo after Monolayer Culture

Abstract. *Mouse mammary tissue grown in primary cell culture did not display specialized characteristics of the parent tissue. When implanted into gland-free mammary fat pads, not only did some of these cultured cells grow and produce normal mammary outgrowths, but some of the outgrowths formed glands which displayed a variety of culture-induced abnormalities.*

The establishment of monolayer cultures from differentiated tissues necessitates a number of changes, notably the immediate loss of tissue-specific structure and the subsequent loss or reduction of identifiable functional activity (*1*). Although Moscona (*2*) has shown that the dissociation of tissues into their component cells does not in itself preclude the reestablishment of histotypic patterns, the ability to form structured aggregates is mainly limited to embryonic cells (*3*). Furthermore, the process of cell culture produces a decline in the ability of dissociated embryonic cartilage cells to reaggregate (*4*).

Functional changes also may occur rapidly. For example, in cultures of embryonic cartilage a progressive reduction in the formation of extracellular cartilaginous matrix occurs until none can be observed after about 10 days (*4, 5*). Bovine mammary tissue has been placed in cell culture by Ebner *et al.* (*6*), who studied the fate of certain mammary-specific biochemical activities. These activities were observed to decline within a few days, but in a nonparallel manner.

In other cases certain characteristics of the differentiated state may reappear after long periods of time in response to changes in the cellular environment. Chick iris epithelium grown as a colorless sheet of cells for a period of a year or more can form pigment after a change in medium (*7*). More recently, Priest and Priest (*8*) have reported that rat fibroblasts grown in culture for 15 transfers without the production of collagen showed biochemical and morphological evidence of collagen formation when placed in diffusion chambers implanted into the rat peritoneal cavity.

These results point up the classic difficulty of determining whether culture-induced changes represent a process of selection, an actual change in differentiative potential, or merely a reversible alteration that is dictated by limitations of the culture situation—alternatives that have been reviewed and discussed by Harris (*9*). One of the principal difficulties in these investigations is the shortage of suitable phenotypic markers. The failure of cells to produce a given structure or display a certain function when studied only in vitro could mean only that the conditions of culture do not favor these activities. The ideal situation in which to test the state of differentiation of cells is that found in vivo—the return of the cultured cells to their site of origin in an isologous animal. Retransplantation of cultured cells into isologous hosts can be employed in the case of neoplastic cells, which may be easily distinguished from host tissues after a period of growth. Cultured normal cells, which are likely to be confused with host material, must be implanted in unusual sites such as the anterior chamber of the eye, unrelated organs, or diffusion chambers—locations which may not allow full expression of differentiative potential.

Some of these difficulties can be avoided by transplanting into the gland-free mammary fat pads of isologous mice according to the techniques of DeOme and Faulkin (*10*). Fragments of mammary gland transplanted into these gland-free fat pads grow and develop into normal mammary structures. At necropsy the glands may be removed, fixed, and stained with hematoxylin; this results in an optically clear structure in which even subtle characteristics of the parenchymal morphology are clearly visible and in which there is virtually no possibility of confusing host with transplanted tissues.

This report concerns experiments in which the transplantation of cultured cells into gland-free fat pads was used in order to measure the effect of primary monolayer culture on the ability of mammary cells to reconstruct normal, functional mammary tissues.

Cell cultures were established by a method based on that of Lasfargues (*11*). The mammary glands were removed from young adult mice of the C57BL/Crgl or BALB/cCrgl strains that were from 14 to 17 days into their first pregnancy. These glands, which showed good lobuloalveolar development but lacked obvious secretion, were finely minced, placed in Hanks solution containing 0.05 to 0.1 percent collagenase, and stirred at room temperature for 90 minutes or until digestion was nearly complete. After pelleting, the cell suspension was pipetted repeatedly until microscopic examination showed only single cells and small clumps containing about one or two dozen cells. The cell suspension was washed, diluted with growth medium in the ratio of 0.08 ml of packed cells per 100 ml of medium, and grown in prescription bottles at 37°C. The nutrient was changed on alternate days and consisted of Medium 199 supplemented with 10 percent newborn or fetal calf serum, insulin (10 μg/ml), penicillin (50 units/ml), and streptomycin (50 units/ml).

During the first week of culture, cells of typical epithelial morphology proliferated until a nearly continuous sheet was formed (Fig. 1*A*). Between the 5th and 7th days fibroblasts became prominent in the spaces not occupied by epithelial cells, but the two cell types were not seen to intermix even in

cultures kept for several weeks. The tubules and acini described by Lasfargues (*11*) were not observed, and morphological evidence of secretion (fat vacuoles in the cytoplasm) disappeared within a few days. Good epithelial growth of this tissue after passage in vitro has not been achieved, and this report is therefore limited to a discussion of cell populations derived from primary cell cultures.

Implantation of cultured cells into the mammary gland-free fat pads of isologous mice was accomplished without the use of enzymes. Bottles were examined microscopically and any remaining cell clumps were located and marked. After a rinsing with Hanks solution, a sample of the cell sheet was scraped from the glass surface by means of the bent tip of a stainless steel wire. A single stroke of about 8 cm in length over the interior face of the bottle yielded a loose aggregate of approximately 5×10^5 cells. The cell aggregate was transferred to a pair of fine forceps and forced into a previously prepared incision in a gland-free fat pad. The usual period of growth in vitro was 6 to 21 days, although cultures up to 6 weeks of age have been transplanted. The usual period of growth in vivo was 10 to 14 weeks.

Several experiments utilizing these techniques have demonstrated that approximately one-half (265/501) of the cell-culture transplants formed outgrowths that were identifiable as mammary tissue under the dissecting microscope. The remaining transplants either could not be located in this way or, if located, failed to show evidence of organization into mammary tissue.

The outgrowths from cultured cells were often normal in appearance (Fig. 1*B*) and resembled closely the 104 control outgrowths that arose from transplanted fragments of uncultured mammary glands or from aggregated samples of collagenase-dissociated but uncultured glands. Except for the absence of a nipple connection, these outgrowths were comparable to the hosts' own mammary glands.

The ability of these outgrowths to complete the cycle of mammary development has been tested. In addition to the formation of typical end buds and ducts in virgin hosts, the outgrowths displayed normal lobuloalveolar development in response to pregnancy. During lactation these lobules became secretory and milk-engorged ducts appeared.

In addition to these growths of normal type, several transplants have produced outgrowths that differ markedly from the normal pattern. These culture-induced abnormalities may be grouped into three categories, as follows.

1) *Hyperplastic.* A few (5 percent) of the outgrowths arising from cultured cells have demonstrated a degree of lobuloalveolar differentiation in virgin hosts. The differentiation may be extensive, involving a significant amount of the glandular outgrowth (Fig. 1*C*). Histologic sections of these outgrowths revealed great variations from normal morphology, ranging from almost normal lobules and alveoli to atypical structures in which differentiation appeared to be imperfect or incomplete. Atypical outgrowths were not formed by uncultured transplants derived from young donors of the low mammary-tumor strains BALB/c and C57. These hyperplastic outgrowths bear some resemblance to outgrowths arising from transplants of hyperplastic alveolar nodules found in the high mammary tumor C3H strain. These nodules are found in glands of old, multiparous C3H females. The transplantation of nodules into cleared fat pads of isologous mice yields hyperplastic outgrowths which, in turn, give rise to mammary tumors (*10*). The tumor-producing capability of the culture-induced hyperplasias derived from the low mammary-tumor BALB/c and C57 strains has not been tested.

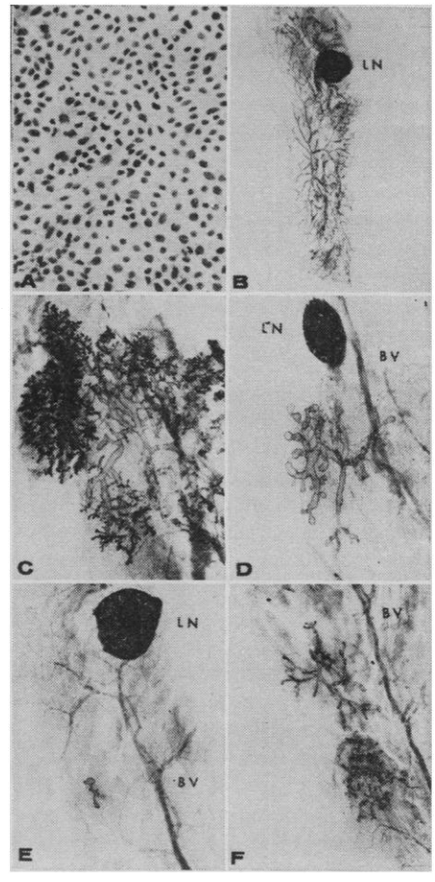

Fig. 1. All photographs of glands (*B* through *F*) show No. 4 mammary fat pads containing mammary gland outgrowths. All fat pads were removed from host mice 3 months after transplantation of cultured cells and stained with hematoxylin. Nonmammary structures present in the fat pads are lymph nodes (*LN*) and blood vessels (*BV*). (*A*) Representative area from 7-day culture of mouse mammary epithelium, hematoxylin-eosin stain (× 90). (*B*) Normal mammary outgrowth from cells 4 weeks in culture (× 2⅔). (*C*) Mammary outgrowth containing a dense hyperplastic area. Transplanted from a 2-week culture (× 4½). (*D*) Mammary outgrowth from a 4-week culture. The ducts are dilated and lack normal end buds (× 4½). (*E*) Outgrowth from cells cultured for 5 weeks. Outgrowth organized into mammary epithelium but failed to fill a significant amount of the fat pad (× 4½). (*F*) Fat pad containing two focuses of glandular outgrowth. Although both outgrowths arose from the same 2-week culture they apear to have different patterns of growth (× 4½).

2) *Ductile abnormalities.* Several (15 percent) of the outgrowths from cultured cells displayed aberrant ductile development that did not involve the formation of alveoli. Some of these outgrowths contained distended ducts, or unusual patterns of branching, or both (Fig. 1*D*). In other instances a complex network of intertwined ducts suggested an abnormal regulation of interductile spacing (*12*). A common type of abnormal outgrowth was characterized by ducts that ended abruptly, without the structured end buds that are normally present at the ends of ducts that have not reached the limits of the fat pad. Finally, some outgrowths consisted of very fragile, atrophic ducts that gave evidence of degeneration or regression.

3) *Slow growth.* Most of the outgrowths derived from uncultured transplants grew to the limits of the fat pad within the period of 10 to 14 weeks. In contrast, 64 percent of the cultured transplants filled less than half of the available area of the fat pad during this period of time, making this by far the most common abnormality encountered in these experiments (Fig. 1*E*). Occasionally, histologic examination was required to identify these small outgrowths. In one experiment transplants were allowed to remain in the

host animals for 14 months, but several fat pads remained only partially filled.

It appears that the transplantation of cultured cells into cleared mammary fat pads represents a technique that allows the expression of both the normal and the abnormal differentiative potential of cultured cells. Unfortunately, there is great variability between and within cultures, although every attempt is made to maintain uniformity. Variation within a single bottle is illustrated in Fig 1*F*, where two outgrowths of different character have arisen from one stroke of the scraping instrument. At present there is no obvious correlation between observable characteristics of the cultures and the nature of the outgrowths derived from these cultures.

Possibly the occurrence of a variety of abnormal growth patterns can be explained on the basis of a selection in culture of one of several minority cell types. According to such a selection hypothesis, the abnormal cells must have existed in the normal, uncultured mammary tissues but were unable to express their potential under the usual conditions of transplantation and growth in vivo. It is also plausible to assume, however, that although many of the normal differentiated properties of the cultured cells reappear after transplantation, other properties are deleted (slow growth) or added (hyperplasia).

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Photosynthetic Phosphorylation: Stimulation by Pteridines and a Comparison with Phosphodoxin

Abstract. *A number of pteridine derivatives stimulate photosynthetic phosphorylation in spinach chloroplasts. In general, tetrahydro or dihydro compounds are highly active, as is one aromatic, naturally occurring compound, biopterin. The physiological characteristics of this photosynthetic phosphorylation are the same as those described for phosphodoxin. A pteridine-containing fraction from spinach, with the same* R_F *value as phosphodoxin, is also active.*

A factor which stimulates photosynthetic phosphorylation in broken chloroplasts of spinach was isolated by Black *et al.* and named "phosphodoxin" (*1*). The published ultraviolet spectrum of this substance is similar to that of a 6-alkyl-2-amino-4-hydroxypteridine. Compounds of this type (in the reduced form) are present in relatively large amounts in blue-green algae (*2, 3*) and to a lesser extent in photosynthetic bacteria (*3*); furthermore, in the case of the blue-green alga *Anacystis nidulans*, cold shock causes the release of pteridines and the concomitant loss of photosynthetic ability and viability (*4*).

These observations led us to test a number of pteridines for their ability to stimulate photosynthetic phosphorylation in broken chloroplasts of spinach. Here we report on the activity of several of the compounds tested and describe briefly the characteristics of pteridine-stimulated photophosphorylation especially with respect to its similarity to phosphodoxin-stimulated photophosphorylation.

The first compound shown to be active was 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (TDP), the synthetic compound used by Kaufman to replace the natural cofactor for phenylalanine hydroxylation (*5*). Activity with TDP was used as a standard in further experiments with naturally occurring compounds. Spinach chloroplasts were isolated according to the method of Arnon *et al.* (*6*) except that the preparation medium contained 0.1*M* tris buffer (*p*H, 7.6), 0.35*M* NaCl, and 0.001*M* ethylenediaminetetraacetic acid. Broken chloroplasts were prepared just before use by diluting a dense suspension of whole chloroplasts with distilled water. Photophosphorylation was measured by the method of Avron (*7*), used also by Black *et al.* (*1*).

Photophosphorylation stimulated by TDP was compared in its characteristics with that stimulated by phosphodoxin. The results are shown in Figs. 1 and 2 and Table 1. At saturating concentrations ($10^{-3}M$), TDP promotes a rate of photophosphorylation approximately equal to that observed in oxygen-requiring photophosphorylation with riboflavin-5′-phosphate ($10^{-6}M$) and half that observed with phenazine methosulfate ($10^{-5}M$).

The characteristics of TDP-stimulated photophosphorylation were at least superficially similar to those of riboflavin-5′-phosphate–stimulated photophosphorylation. Other tetrahydropteridines showing similar activity and characteristics were tetrahydrofolic acid, tetrahydrobiopterin glucoside, and 2,4-dihydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

The reported absorption spectrum of phosphodoxin resembles that of an aromatic pteridine, not a tetrahydro derivative. Some aromatic pteridines were therefore tested. Under the conditions described in Fig. 1, the rela-

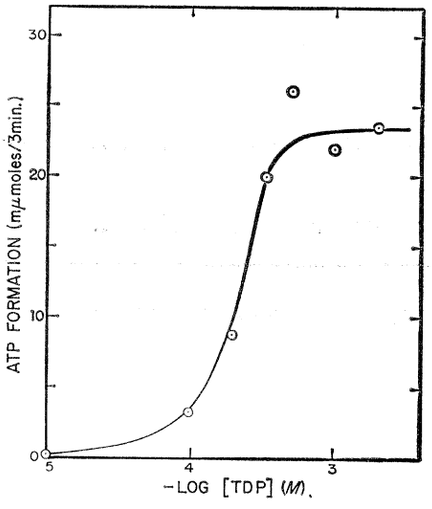

Fig. 1. Rate of formation of ATP with increasing concentrations of TDP. Reactions were carried out in 17-mm tubes containing tris buffer, *p*H 7.4 (4.8 × $10^{-2}M$); $MgSO_4$ (2 × $10^{-3}M$); inorganic phosphate, P_i ($10^{-3}M$); adenosine diphosphate ($10^{-3}M$); $^{32}P_i$ (approximately 0.5 μc); broken spinach chloroplasts (18.8 μg of chlorophyll); these were contained in a total volume of 1.0 ml. Illumination, incandescent light (14,000 lux); incubation, 3 minutes at 26°C. For other experiments mentioned in the text the TDP concentration was $10^{-3}M$.