about 2.5×10^{-2} watts cm^{-3}. If the magnification is raised to 10,000 times, then the area scanned is about 10^{-6} cm^2, and the value of power per unit volume increases to approximately that for a stationary beam. Although these dose rates are quite large, only the outer 10^{-3} cm of the specimen is significantly irradiated.

Consider now the thermal effect of an electron beam current (energy of 25 kev) scanning an area of 10^{-4} cm^2; if this area is (for mathematical convenience) in the form of a concave hemisphere of radius 5×10^{-3} cm, one can calculate the steady state temperature rise θ at this surface. If the heat loss is solely by uniform thermal conduction through the tissue, then θ can be expressed as

$$\theta = \frac{P}{\sigma}\int_{\infty}^{5 \times 10^{-3}} \frac{dx}{2\pi x^2}$$

where P is the beam power, σ is the thermal conductivity of the tissue, and x is the distance into the tissue. In our case P is equal to 25×10^{-8} watt and σ is equal to 20×10^{-4} watt cm^{-1} $°C^{-1}$ [this is the value quoted for wood (*8*); no figures were available for insect tissue]. Hence θ is equal to 0.005°C. This increase should not be troublesome; the fact that the increase is so small may be one reason why scanning electron-micrographs can be taken of living specimens, apparently without serious effects caused by electron irradiation.

The current or the energy of the electron beam can be raised, and hence it should be possible to study the biological effects of irradiation on selected areas as small as 10^{-6} cm^2 in living specimens of the various developmental stages of *Tribolium*.

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18 October 1966

Establishment of Four Functional, Clonal Strains of Animal Cells in Culture

Abstract. *The single-cell plating technique was used to develop four clonal cell lines that perform organ-specific functions after being serially cultured for prolonged periods. These strains include steroid-secreting Leydig cells, melanoma cells that form pigment, and two strains from a hormone-secreting rat pituitary tumor. One of the cell lines from the pituitary tumor secretes growth hormone, while another line derived from the same tumor secretes a substance similar to adrenocorticotropic hormone.*

The loss of organ-specific function is commonly observed in cultured animal cells during serial propagation (*1*). This loss has been ascribed to (i) selective overgrowth by connective tissue cells, (ii) a phenotypic change in the cultured cells, or (iii) inadequate or harmful environmental conditions (*1*). By using the single-cell plating technique to eliminate the possibility of selective overgrowth, we have established four new clonal cell strains. Even after having been continuously cultured serially for a period of up to 1 year, each cell line performs at least one of the major organ-specific functions of the tissue from which it was derived.

Cultures were established by the method of alternate culture and animal passage (*2*). Primary cultures were made from three transplantable animal tumors that retain differentiated function after serial transfer in animals: (i) a rat pituitary gland tumor, MtT/W5 (*3*); (ii) a tumor of Leydig cells from mouse testicle (*4*); and (iii) a mouse melanoma (*5*). After short periods (3 to 10 days) in culture, the surviving cells from each type of tumor were harvested and injected into appropriate host animals. In this way new tumors were obtained, and these, in turn, were placed in culture. This process was repeated from three to six times with each tumor. As Buonassisi *et al.* had previously shown for adrenal tumor cells (*2*), these new culture-derived tumors also had an enhanced ability to survive and grow in vitro. The rationale for this approach is that the initial culture periods select for cells that can withstand the conditions of culture. A further advantage of this method is that it does not select for supporting tissue cells, which usually overgrow the desired cell type. Since the connective tissue cells are not malignant, they would not be expected to survive grafting and would not contribute to a new tumor. Cultures were grown in plastic petri dishes (60 by 15 mm) and incubated at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air. Synthetic medium F10 (*6*) was supplemented with 15 percent horse serum and 2.5 percent fetal calf serum. The cells were recovered for subculture (at 2-week intervals) by being incubated in 0.1 percent Viokase solution in phosphate-buffered saline for 5 to 10 minutes at 37°C (*7*). Single-cell platings, without feeder layer, were made from each of the cultures by the method of Puck, Marcus, and Ciecura (*8*). Stainless steel cylinders were used for the isolation of individual colonies. The isolated colonies were detached and dispersed into single cells with Viokase solution. The dispersed cells were counted, serially diluted, and distributed in individual petri dishes. Plating efficiencies of 5 to 10 percent were regularly obtained. Original clonal strains were recloned, in some instances more than three times, and no morphological or functional differences between the parent and derived clonal strains were seen.

We determined differentiated function of each clonal cell line by examining the cells or the culture medium for a product characteristic of the tissue from which the cells were derived. We tested the medium from the cultures of pituitary cells for adreno-

corticotropic hormone (ACTH) by measuring its effect on steroidogenesis by clonal adrenal cells (*7*). The adrenal cells secreted Δ^4-3-ketosteroids into the culture medium (*2*). ACTH added to the medium of adrenal cells greatly increased the biosynthesis and secretion of these steroids, which have a characteristic ultraviolet absorption spectrum with a maximum at 242 mμ (*2*). The concentration of Δ^4-3-ketosteroids was measured spectrophotometrically on extracts of the medium from adrenal-cell cultures by a previously described method (*2*). Steroid secretion into the culture medium was related linearly to the logarithm of the amount of ACTH added [0.1 to 10 milliunit (International Unit) per plate] and, therefore, formed the basis of a sensitive bioassay for ACTH. We determined the ability of pituitary cells to secrete growth hormone by measuring the striking increase in body weight and linear growth that occurred in intact and hypophysectomized rats injected with these cells. The secretion of Δ^4-3-ketosteroids by Leydig cells was measured spectrophotometrically on extracts of the culture medium prepared according to methods described for adrenal cells (*2*). Pigment formation by melanoma cells was seen by direct microscopic observation of the unstained cells. The degree of pigmentation varied from cell to cell in the clonal cultures as has been previously reported by Hu (*9*). Dispersed cells were counted in a hemocytometer. The nitrogen concentration of cultured cells was determined by the method of Lowry *et al.* (*10*).

The primary culture of the rat pituitary tumor was morphologically heterogeneous (Fig. 1a). Clonal lines of epithelial cells (Fig. 1b) and flat, spindle-shaped cells (Fig. 1c) were established. The two clonal cell lines were usually subcultured at 2 to 3 week intervals and divided either 1 to 4 or 1 to 8. Clonal epithelial cells and clonal flat cells were each gently scraped from the plates with a rubber policeman. The cells were suspended in isotonic NaCl and injected subcutaneously into intact or hypophysec-

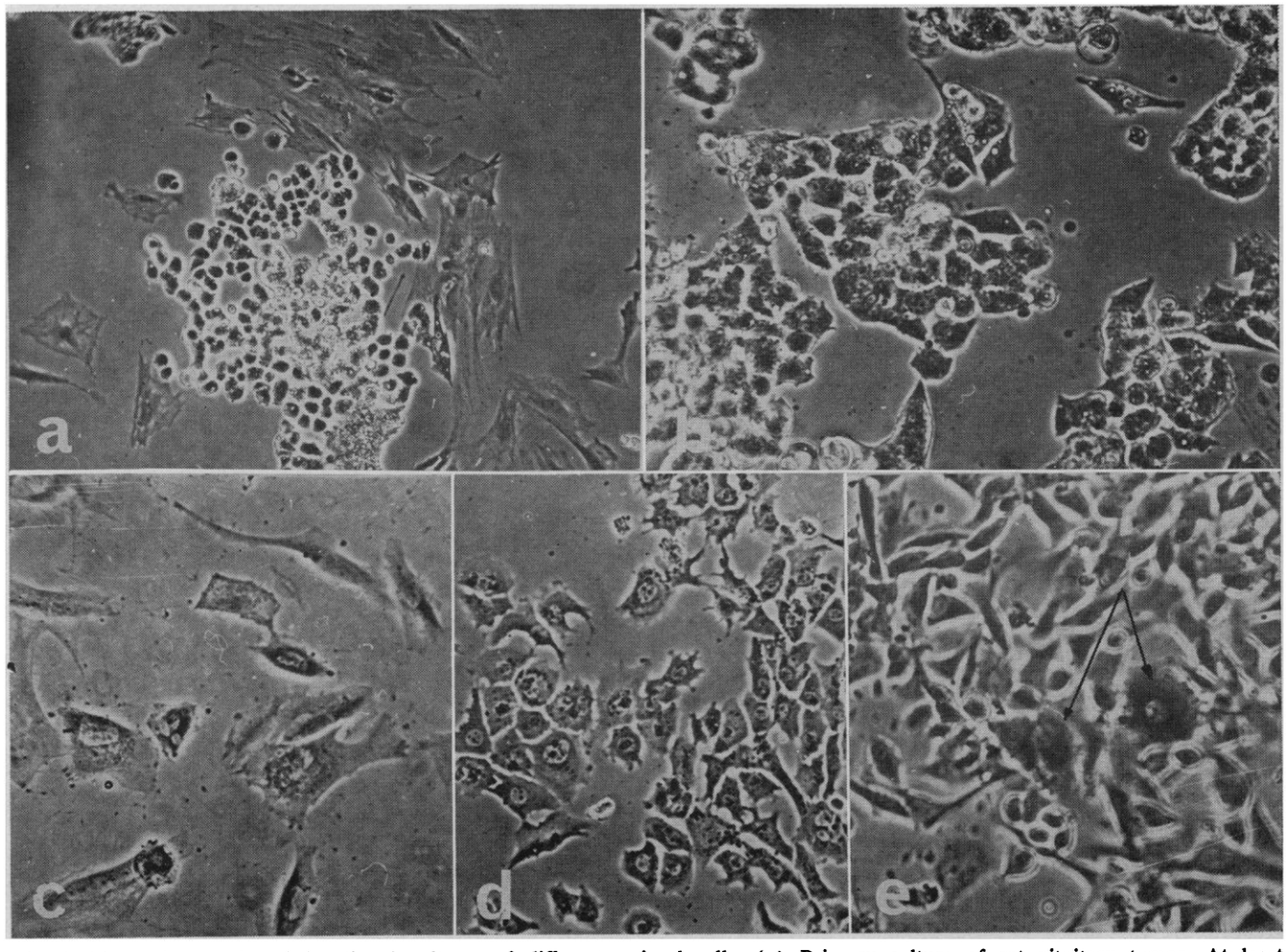

Fig. 1. Photomicrographs of functional cultures of different animal cells. (a) Primary culture of rat pituitary tumor. At least two morphologically distinct cell types can be seen—epithelial cells and fibroblast-like cells. On close, repeated examination we could divide the fibroblast-like cells into two subgroups—typical fibroblasts and much larger, very flat, irregularly shaped cells (called "flat cells," Fig. 1c). (b) Clonal epithelial-cell line derived from rat pituitary tumor cultures. These cells secrete growth hormone when injected into rats of the same strain (Fig. 2). (c) Clonal flat-cell line derived from rat pituitary tumor cultures. These cells secrete ACTH-like material into the culture medium (Fig. 3). (d) Clonal Leydig cells obtained from mouse testicular tumor. (e) Clonal cell line derived from mouse melanoma. Scattered areas of dark pigment accumulation (arrows) can be seen in the cells of these cultures (Magnification $\times$ 70 to 140; fresh, unstained cultures).

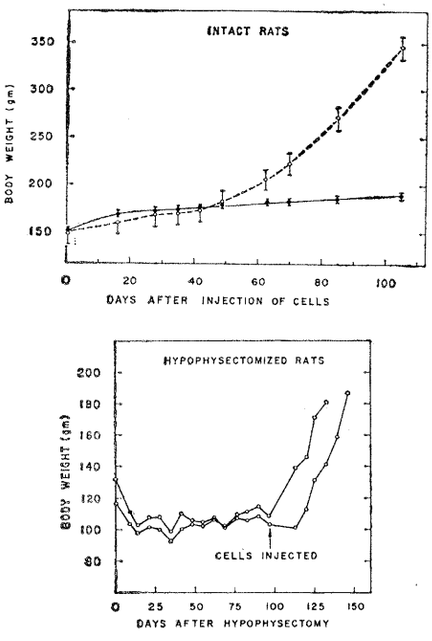

Fig. 2 (left). Effects on body weight of the injection of clonal, pituitary, epithelial cells subcutaneously into intact (top) or hypophysectomized rats (bottom). All rats were females of the Wistar-Furth strain. Intact rats (upper panel) were each injected on day zero with approximately 4 million cells that had been in continuous serial culture for 8 months. Each point gives the mean body weight of four injected rats (○) or 6 uninjected control rats (●). The vertical lines give the standard error. The lower panel shows the effects on body weight of injection of approximately 12 million cells into each of two rats 97 days after hypophysectomy. Hypophysectomized rats had free access to thyroxine (2 μg/ml) in the drinking water. The body weight gain that occurred following injection of clonal epithelial cells was accompanied by a parallel increase in linear growth (tip of nose to tip of tail) in both intact and hypophysectomized rats. The tumor-bearing rats were usually 3 to 5 cm longer than the control rats at the same time their body weights were twice the control level.

tomized rats. Epithelial cells in continuous culture for periods up to 1 year invariably produced tumors in recipient rats within 8 to 10 weeks, even when as little as 300 μg of cell nitrogen was injected into each rat. The appearance of tumors was followed by a marked increase in body weight (Fig. 2) and stimulation of linear (nose-to-tail) growth in all animals. Similar results were obtained in eight consecutive experiments in which at least three rats were injected in each trial. In five experiments tumor-bearing and control rats were sacrificed when the tumor-bearing animals were about one and one-half to two times as heavy as control rats of the same age (306 to 470 g as compared to 177 to 210 g). The excised tumors from 12 rats ranged in weight from 2 to 54 g with a mean value of 15 g. The weight of tumor tissue never exceeded 20 percent of the total weight gained (above control); in most of the rats the tumors weighed less than 10 percent of the total weight gained. No tumors were detected in rats injected with clonal flat cells (up to 8 months after injection) despite the administration of as many as 20 million cells per rat. Rats injected with flat cells did not differ from control rats with respect to rate of weight gain or linear growth. The lack of significant weight gain over periods of up to 100 days after removal of the pituitary gland was taken as evidence for complete hypophysectomy in the rats shown in Fig. 2 (bottom) and in other, similar groups of hypophysectomized rats. We obtained evidence supporting this conclusion at the end of these experiments by examining, under a dissecting miscroscope, the sella turcica, and the surrounding area, of each hypophysectomized rat. No residual pituitary-like tissue was seen in any of the rats reported here. Therefore, it is unlikely that the weight gain in tumor-bearing rats is due to secretion by the tumor of a substance which stimulates synthesis and release of growth hormone by the pituitary gland. We interpret the gain in body weight and stimulation of linear growth that occurred in intact or hypophysectomized rats injected with clonal epithelial cells as evidence that these cells secrete growth hormone in the recipient animals. In addition, it has recently been shown by two different immunological methods that these clonal epithelial cells secrete large amounts of growth hormone into the culture medium (*11*). The established, clonal, pituitary epithelial-cell line appears, therefore, to carry out organ-specific function both in vitro and in the host animal after more than 1 year in continuous culture.

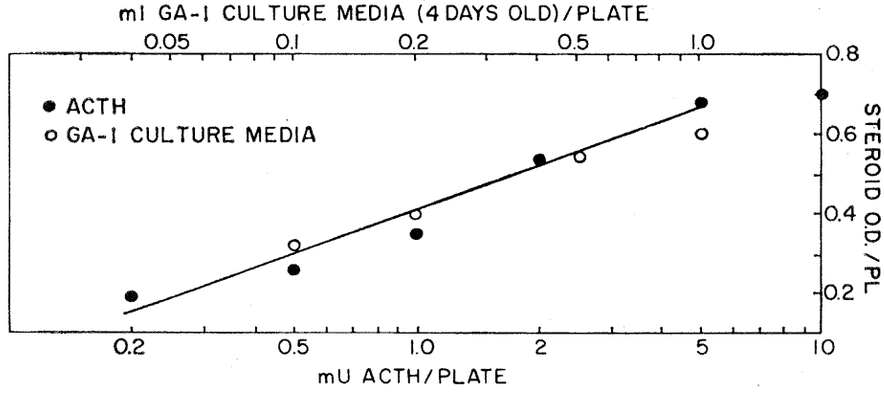

Fig. 3. Effects of porcine ACTH (●) and culture medium from clonal, pituitary flat cells (GA-1 culture media, ○) on steroid hormone production by adrenal cells. The ordinate gives the optical density at 242 mμ of the final ethanol extract of adrenal-cell culture media harvested after 24 hours of incubation with ACTH (0.2 to 10.0 milliunit per plate, ACTH being standardized against the latest international standard) or with media (4 days old) from clonal flat-cell cultures (0.1 to 1.0 ml per plate). Each point is the mean value determined from two adrenal-cell plates. The log dose response to the flat-cell culture media (obtained after the cells had been in serial culture for 107 days) was linear and parallel to the response given by ACTH. The ACTH-like material accumulated in the medium of flat-cell cultures at a rate of 2 to 5 milliunit per milligram protein in 24 hours. Media from clonal, pituitary epithelial-cell cultures, even at a dose of 1.0 ml per plate, did not stimulate steroid production by adrenal cells.

Medium (3 or 4 days) obtained from epithelial-cell cultures did not stimulate steroidogenesis by clonal adrenal cells even at a dose of 1.0 ml per test plate (*2*). Medium (3 or 4 days) from flat-cell cultures, however, markedly stimulated steroidogenesis at a dose of 0.1 ml per test plate and gave a response that was linear and parallel to that obtained with authentic, porcine ACTH when this response was plotted as a function of the logarithm of the dose (Fig. 3). These results are evidence that the clonal flat cells, after 107 days in continuous culture, secrete into the culture medium ACTH or a substance with biological properties very similar to ACTH. Clonal

flat cells, after more than 200 days in culture, continue to secrete ACTH-like material into the medium; however, the log dose response to flat-cell culture medium is no longer parallel to the response obtained with porcine ACTH. The meaning of this change is not yet known. The material secreted by cells in culture more than 200 days may be a different compound from that secreted by the early cultures; it may not be free, native ACTH; it may not be an ACTH-like peptide at all, but another substance that stimulates steroidogenesis by adrenocortical tumor cells.

Clonal, testicular Leydig-cell cultures (Fig. 1d) secreted Δ^4-3-keto-steroids into the medium at a rate of 1.0 μg/mg of protein per hour after 12 months in continuous culture. Dark brown pigment was formed by clonal melanoma cells maintained in culture for over a year (Fig. 1e).

Our experiments show that serially-propagated clonal strains of animal cells can perform specialized, organ-specific functions for prolonged periods. The marked stability of functional properties after cloning implicates selective overgrowth as a major factor in the loss of specialized function in culture. Nearly limitless numbers of isolated functional cells for physiological, biochemical, and embryological experiments can thus be obtained by the combination of alternate animal and culture passage and cloning techniques.

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Germination of Witchweed (Striga lutea Lour.): Isolation and Properties of a Potent Stimulant

Abstract. *A crystalline germination stimulant (trivial name strigol) for the root parasite, witchweed* (Striga lutea *Lour.), has been isolated from cotton root exudates and characterized as a $C_{19}H_{22}O_6$ compound. Although apparently different from known plant hormones, the stimulant is active at hormonal levels, causing germination at concentrations less than 10^{-5} part per million.*

Witchweed (*Striga lutea* Lour.) (*1*) is an angiospermous root parasite indigenous to several tropical and subtropical areas of the Eastern Hemisphere. The discovery of *S. lutea* in the coastal plain section of the Carolinas in 1956 has necessitated a costly quarantine and control program (*2*), and its depredations in the food-poor countries of the Eastern Hemisphere have long been a source of concern. With few exceptions, seeds of the parasite [and related parasites such as *Striga hermonthica* (Del.) Benth] remain dormant until germination is stimulated by a chemical produced by the host plant or by certain other plant species (*3*). Identification of the stimulant and an understanding of the germination process seem basic to a rational control program. In addition, this information could provide an opening into fundamental knowledge regarding seed germination in general. The identity of the stimulant (or stimulants) has been the object of considerable research (*4*), but the low concentrations in which it is produced and its relative instability have hitherto prevented isolation in pure form.

We wish to report the isolation of a crystalline, highly active germination stimulant for *S. lutea* from cotton root exudates. Cotton plants were grown hydroponically and the nutrient fluid periodically circulated through charcoal columns. The charcoal saturated with stimulant was eluted with acetone, and the resulting aqueous acetone solution was evaporated at reduced pressure to leave an aqueous solution of the stimulant (*4*). Extraction with benzene at this point gave a 30-fold concentration of active material in the organic layer. The residue from evaporation of this layer was separated by preparative thin-layer chromatography on silica gel into two stimulatory substances, *A* and *B*, which were purified by further chromatography. Crystallization of *A* from acetone-hexane or benzene-hexane gave pure *A* as white needles. We propose the trivial name strigol for this compound.

The stimulant activity of purified materials was compared by determining their effect on the germination of *S. lutea* seeds pretreated with water (*5*). Crystalline strigol produced 50 percent germination at concentrations of less than 10^{-5} part per million in water, and some noncrystalline preparations of stimulant *B* were at least as active.

The melting point of strigol varies somewhat with the rate of heating, but the purest sample melted at 200° to 202°C with decomposition after some softening around 195°C (Kofler apparatus). It is readily soluble in moderately polar solvents (for example, acetone, methylene chloride), moderately soluble in benzene, and relatively insoluble in hexane.

The infrared spectrum of strigol in methylene chloride (Fig. 1) has bands at 3590, 1787, 1745, 1682, and 1601 cm^{-1}; the 1787 band is the most intense. In samples crude enough to be soluble in carbon disulfide, the two high-frequency carbonyl bands appear at 1795 (more intense) and 1755 cm^{-1}, whereas in chloroform they shift to 1786 and 1740 cm^{-1}, and the 1740 cm^{-1} band is the more intense. There is an ultraviolet maximum at 236 mμ (molar extinction coefficient about 18,000). Prominent features indicated by nuclear magnetic resonance spectra (*6*) are two methyl groups (at 1.16 and 1.08 δ from internal tetramethylsilane), a methyl or methylene group attached to an unsaturated system (broadened singlet at 2.00 δ), and a highly deshielded hydrogen (7.42 δ).