ions on either side of the membrane, although the action potential is prolonged. Changes in the concentration of H, Ca, or Mg ions may block conduction. The resting and action potentials are somewhat sensitive to Na-ion concentration. These effects are consistent with a model that emphasizes the multilayer structure of the nerve membrane and the divalent ions therein.

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*HORMONE-PRODUCING CULTURES OF ADRENAL AND PITUITARY TUMOR ORIGIN**

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Animal cell cultures rarely perform the differentiated functions of the tissue of origin for any practical length of time. This has been especially true of cultures of endocrine tissue although a few cultures of endocrine origin have been reported to maintain their function.[1] The present work deals with a systematic procedure which may prove to be generally applicable for developing cultures of physiologically specialized cells which maintain these specialized functions in culture. To this end, hormone-producing mouse tumors were put into culture, and after various times in culture, the cultures were injected into mice to obtain new tumors. Tumors arising from cultures were checked for hormonal activity and put back into culture, and the whole process was repeated. It was hoped that this process would selectively enrich the tumors for cells better able to withstand the conditions of culture. Furthermore, only the cancerous endocrine cells would benefit from this selective process because stromal elements which survive the culture period should not participate in the process of new tumor formation. The experiments indicate that successive passages of these tumor cells through culture and animal give rise to cells with increased growth capacity and enhanced hormonal activity in culture.

Materials and Methods.—The functional adrenocortical and ACTH-secreting mouse tumors used in this study have been developed and extensively investigated by Furth and his associates.[2-7] The culture techniques have been previously described.[8, 9] The only modification adopted here was that the disaggregated tissue was incubated in 1% Viokase‡ solutions in physiological saline for a few minutes at room temperature.

The assay of delta 4-3-ketosteroid secretion by the adrenal tumor cultures was performed in a manner similar to that previously described for tumor slices.[5] The medium was aspirated from the cultures and replaced with 2.0 ml of incubation medium per petri dish, containing a determined amount of ACTH. The incubation medium was either Krebs-Ringer bicarbonate buffer fortified with 200 mg per cent glucose or medium 199 plus 5% horse serum. The two incubation media gave equivalent results for short incubation periods (0–4 hr), but for long (4–24 hr) incubations, medium 199 plus horse serum was superior. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air for a period ranging from 2 to 24 hr. The rate of steroid secretion was found to be constant over the 24-hr period. The incubation medium was extracted with a two-fold volume of methylene chloride (spectrophotometric grade), evaporated to dryness under nitrogen, and dissolved in 95% ethanol. The optical densities of the alcoholic solutions were then measured on the Cary spectrophotometer between 300 and 200 millimicrons. A blank was prepared in the same way as the sample from fresh incubation medium. Quantitation of steroid secretion was obtained by comparing the optical density of the samples at the characteristic peak absorption (242 millimicrons) with those obtained with standard solutions of cortisol. The ACTH used in these studies was obtained from the Armour Company (40 I.U./vial). U.S.P. reference standard ACTH gave equivalent results for short incubation periods but seemed to be toxic over long periods. Steroid production by these cultures was found to be proportional to the logarithm of the dose of ACTH. Estimates of the amount of ACTH in pituitary tumor cultures were made by stimulating adrenal tumor cultures with the pituitary tumor culture media and comparing the steroid production to that obtained with known amounts of Armour ACTH.

Antisera were prepared as previously described.[8] Antisera against normal mouse adrenal cortex were absorbed with homogenates of mouse lung, kidney, liver, and abdominal muscle to remove nonspecific antibodies.

Protein content of the tumors and culture cells were determined by the method of Lowry.[10]

Results.—(1) *Effect of previous culture passage on growth of tumors in culture:* The growth capacity of adrenal tumors put in culture for the first time was compared to that of tumors arising from previous cultures (Fig. 1). The two adrenal tumors were plated at a level of 10^5 cells per petri dish. Under these conditions, almost 100 per cent of the inoculated cells become attached and stretched on the petri dish surface. It is seen that the original adrenal tumor, when put into culture, gives rise to a cell population which does not increase in number with time, but in fact, slowly declines. On the other hand, the adrenal tumor which had been previously passed through culture increases by a factor of ten in about ten days of culture.

The pituitary tumor also exhibits this behavior. When dispersed cells from a pituitary tumor which had never been in culture previously are plated, less than 0.1

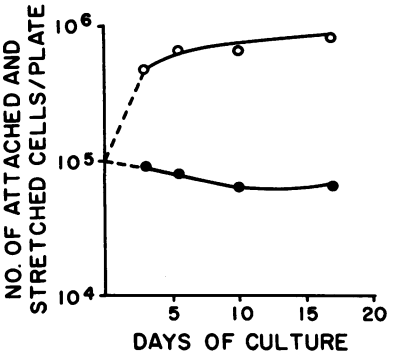

FIG. 1.—Effect of previous culture passage on growth of tumors in culture. 10^5 cells of the original adrenal tumor were plated per petri dish, and the number of attached and stretched cells per petri dish was measured as a function of time in culture (lower curve). The upper curve represents the growth obtained when the same number of cells from an adrenal tumor which had been previously cultured, in this case once for thirty days and subsequently for three days, was plated.

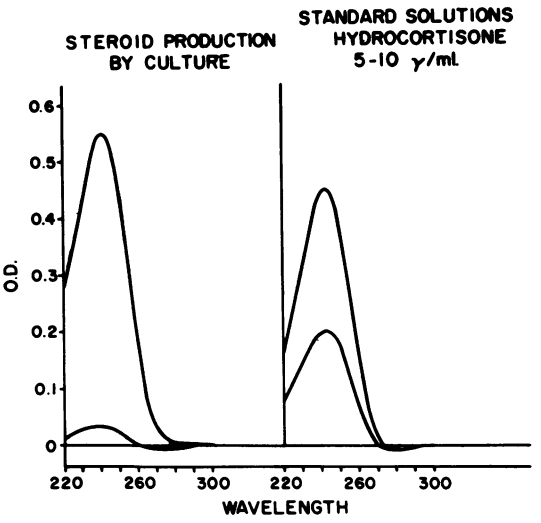

FIG. 2.—Steroid production by adrenal tumor cultures. Absorption spectra of steroidal material obtained from adrenal tumor cultures are presented on the left. The upper curve was obtained upon maximal stimulation of the cultures with ACTH and the lower curve in the absence of ACTH. The curves on the right represent the absorption spectra of hydrocortisone solutions. The adrenal tumor cultures used in this experiment were derived from a tumor which had been in culture twice previously (once for 45 days and then for 27 days).

per cent of the inoculated cells attach, stretch, and initiate growth; for pituitary tumors arising from injection of cultures, this figure is close to 100 per cent.

(2) *Hormonal activity of tumors arising from cultures:* After the first passage through culture, slices of adrenal tumor usually have a greater capacity (1.3–1.8 gamma steroids/mg protein/2 hr) for steroid production than slices of the original tumor (about 1.1 gamma steroids/mg protein/2 hr). Pituitary tumors also have enhanced hormonal activity after the first passage through culture. The original pituitary line has an ACTH content of 25–50 mu ACTH/mg tissue, while a tumor arising from culture has a content greater than 400 mu ACTH/mg tissue.

(3) *Immunologic characterization of culture populations:* In the early stages of this work, the production of steroids in culture by the original adrenal tumor was barely and not constantly detectable. If only a small fraction of the cells in culture were of adrenal cortex origin, the low production of hormones by the cultures could be accounted for. To get a more precise description of the cell population of these cultures, four-week-old cultures of adrenal tumor and 4-week-old control cultures of mouse lung and pituitary tumor were treated with anti-adrenal cortex antiserum as shown in Table 1. It is seen that mouse lung and mouse pituitary tumor cultures are relatively unaffected over the range of antiserum used, while mouse adrenal tumor cultures are destroyed even at the lowest concentration of antiserum used. It appears that the adrenal tumor cultures are composed almost exclusively of cells containing antigens specific to adrenal cortex, and therefore, population hetero-

TABLE 1

TREATMENT OF CULTURES WITH ANTI-ADRENAL CORTEXT ANTISERUM

Treatment	4-week-old culture of mouse lung	4-week-old culture of pituitary tumor	4-week-old culture of adrenal tumor
	(Number of attached and stretched cells per petri dish)		
No antiserum	10,020	2,033	7,014
6% anti-adrenal cortex antiserum	9,202	1,980	211
10% anti-adrenal cortex antiserum	16,664	—	3
25% anti-adrenal cortex antiserum	9,208	522	1
6% anti-adrenal cortex antiserum(unabsorbed)	57	20	0

Cultures were trypsinized and plated in the presence of various concentrations of anti-adrenal cortex antiserum and 1 to 2 $C'H_{50}$ units of guinea pig complement/ml. Antisera were absorbed with homogenates of mouse liver, lung, kidney, and abdominal muscle. After 12 hr of incubation, plates were fixed, stained, and scored for number of attached and stretched cells/plate.

geneity can probably be excluded as an explanation for the low production of steroids by tumors in culture for the first time.

(4) *Steroid production by adrenal tumor cultures:* Figure 2 illustrates the procedure used to detect the production of steroids by cultures of adrenal tumors. Cultures were incubated with and without ACTH as described in the section on *Methods*. The typical absorption spectrum for adrenal delta-4-3-ketosteroids is obtained when the cultures are stimulated with ACTH, while a smaller but still distinct peak representing basal secretion is obtained in the absence of added ACTH. Extraction of the culture cells themselves from replicate cultures prior to ACTH stimulation does not yield any steroid-like material. The steroids found in the incubation buffer must, therefore, represent synthesis rather than release of preformed material.

The steroids secreted by these cultures as analyzed by paper chromatography yielded a spectrum similar to that observed earlier in the case of tumor slice secretion,[7] with the exception that 11-OH-androstene-3,17-dione, formerly a major steroid component was now definitely absent. An unidentified steroid (Compound VI), which was produced in increasing quantities by tumor slices following successive tumor transplantations, was now the major steroid component of the adrenal cell culture secretion. On the basis of paper chromatographic characteristics, this compound is probably a hydroxylated progesterone derivative with two hydroxyl groups on the molecule, but without hydroxylation at C_{21}. Three minor steroids were also observed on the paper chromatogram as was the case with adrenal tumor slice incubation extracts.

(5) *Culture growth and steroid production:* In order to know how the specific activity of steroid production is maintained as the culture grows, the experiment presented in Figure 3 was performed with an adrenal tumor arising from injection of a 45-day old culture. An unexpected finding was that the specific activity of steroid production (measured as gammas steroid/mg protein/2 hr) of cultures was six times as great as the specific activity of slices of tumor from which the culture was derived. It is seen that the cells can multiply with maintenance of the specific activity of steroid production. However, as the plates become overgrown, the specific activity begins to decline, in this case by a factor of two. Upon subculture, steroidogenesis becomes undetectable and reappears after some time to an ex-

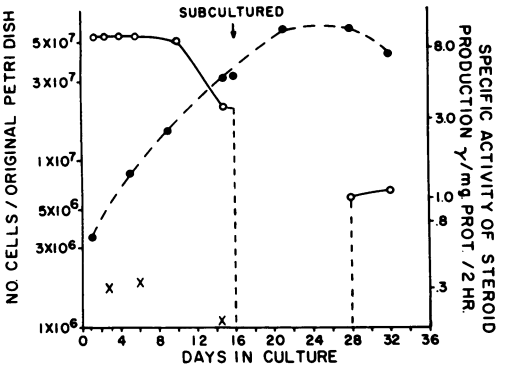

FIG. 3.—Culture growth and steroid production. Growth (solid circles) and the rate of steroid production under maximal ACTH stimulation (open circles) were followed for 34 days for one adrenal tumor culture derived from a tumor previously passaged through culture. After subculture, steroid production is not detectable for a variable length of time as indicated by the dashed line. The X's represent the specific activity of steroid production of an adrenal tumor which had never been in culture previously.

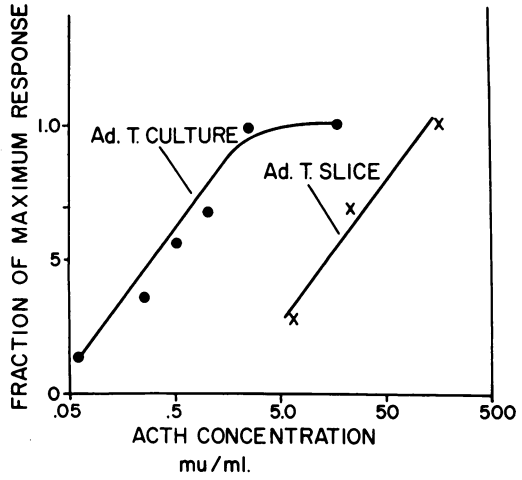

FIG. 4.—ACTH dose-response relationship. Adrenal tumor cultures and adrenal tumor slices were stimulated with graded doses of ACTH and the steroid response measured. The cultures and the tissue slices were derived from the same tumor. The relationship between response and ACTH dose is the same for slices and cultures but higher ACTH doses are required for stimulation of the slices.

tent which is variable. This result is a constant feature of our experiments and is found whether the plates are harvested for subculture by means of trypsin, scraping, or versene. The initial high level of steroid production has been maintained for up to 90 days, but usually the level of steroid production begins to decline after the first month of culture. Cultures which no longer produce any detectable steroids, upon injection into mice, produce tumors with fully restored hormonal activity. Figure 3 also shows that tumors in culture for the first time possess a low specific activity of steroid production.

(6) *ACTH-dose-response relationship:* In the course of the previous experiments, it was found that adrenal tumor cultures are maximally stimulated by a concentration of ACTH as low as 5 mu/plate. On the other hand, it was known that tumor slices require about 200 mu ACTH/100 mg tissue for maximal stimulation. The response of both tumor slices and tumor cultures to graded doses of ACTH is presented in Figure 4. It is seen that the same relationship between response and ACTH concentration holds for both cultures and slices; namely, the response approaches a maximum level at high ACTH concentrations and is proportional to the logarithm of the ACTH concentration at limiting concentrations, but the slices require about 40 times as much ACTH as the cultures to obtain the same rate of steroid synthesis. The response of these cultures to ACTH is apparently specific, since in preliminary experiments, tissue extracts, horse serum, culture media from cultures other than pituitary tumor cultures, and pituitary hormones other than ACTH do not elicit this response.

(7) *Detection and quantitation of ACTH produced by pituitary tumor cultures:* The production of steroids by adrenal tumor cultures upon ACTH stimulation sug-

gested that the system could be used to detect ACTH production by cultures of pituitary tumors. In Figure 5 are presented the results obtained when adrenal tumor cultures are stimulated with graded doses of Armour ACTH and graded doses of pituitary tumor culture medium. Although the maximum response obtained with pituitary tumor culture medium is always higher than the maximum response obtained with Armour ACTH, the same relationship between response and dosage is obtained. This enables us to estimate the amount of ACTH in the culture medium. For this purpose, we assume that the same fractional degree of stimulation is obtained with the same number of units of ACTH. It is not yet known whether the difference between Armour ACTH and mouse pituitary tumor culture medium is due to a species difference between hog and mouse ACTH or to accessory potentiating factors in the culture medium. Unlike pituitary tumor cells *in vivo* which are capable of storing up to 400 mu ACTH/mg tissue, cells in culture seem incapable of storing any ACTH, since ACTH activity has only been found in the supernatant fluid and not in the cells themselves.

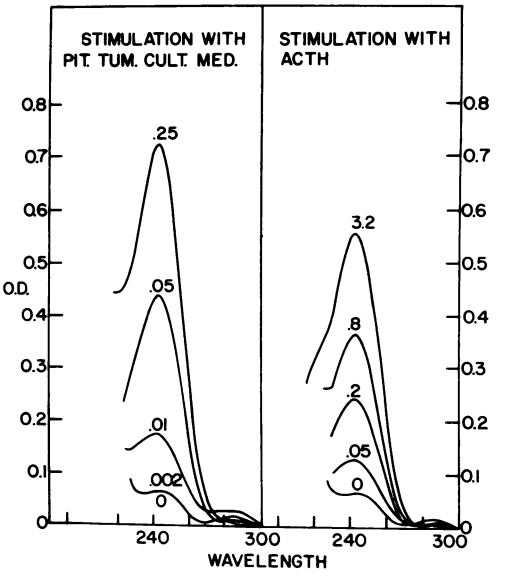

FIG. 5.—Assay of ACTH produced by pituitary tumor cultures. The family of curves on the right represent the absorption spectra obtained when adrenal cultures are stimulated with 0, 0.05, 0.2, 0.8, and 3.2 mμ ACTH per ml incubation medium. The curves on the left were obtained when the adrenal tumor cultures were stimulated with 0, 0.002, 0.01, 0.05, and 0.25 ml of pituitary tumor culture medium per ml incubation medium.

(8) *Growth of pituitary tumor cultures and ACTH synthesis:* Figure 6 illustrates the growth curve of a culture of a pituitary tumor which had previously been in culture for six days and the amounts of ACTH produced per petri dish during various 24-hr periods. As the number of cells increases, the rate of ACTH synthesis increases in a parallel manner. At about the thirtieth day of culture, ACTH production usually begins to decline. However, cultures which have ceased producing ACTH are capable of giving rise to tumors with renewed capacity for ACTH synthesis.

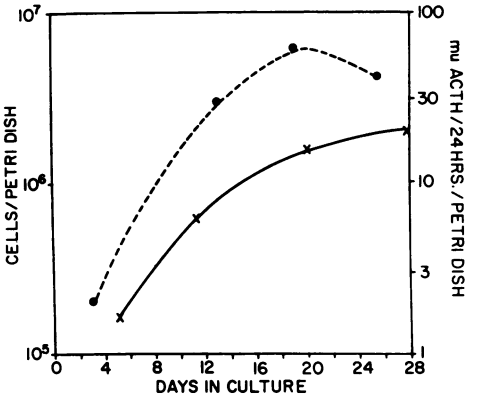

FIG. 6.—Growth of pituitary tumor cultures and ACTH synthesis. The solid curve represents the growth of a pituitary tumor in culture. The dashed curve represents the amount of ACTH produced per petri dish over a 24-hr period.

Discussion.—Since the adrenal tumor cells multiply and maintain steroid secretion for as long as ninety days in culture, it would seem that in their present state of development, the cultures provide a sensitive system both for the study of the mechanism of ACTH action and for the assay of ACTH. The pituitary tumor cultures, which maintain function for several weeks, also provide a convenient system for the study of the mechanism controlling ACTH secretion. Both cultures offer possibilities for study of the factors involved in the loss of specialized function in culture. The adrenal tumor cultures are especially suited for this purpose because considerable knowledge is available on the enzymatic steps involved in steroidogenesis, and the loss of the enzymatic steps can be followed individually.

Although cultures of tumors which no longer show any detectable specific function are still able to give rise to tumors with restored hormonal activity, it is not clear whether cells which have lost this activity regain it *in vivo*, or whether nonproducing cultures still contain a few cells which continue to produce hormones and which are responsible for generating the new tumor cell population. It is hoped that cloning experiments will resolve this question.

Summary.—Cultures of adrenal and pituitary tumor have been obtained by alternate passaging of tumors through culture and animal. This procedure enhances the hormonal activity and growth capacity of these cells in culture. Cultures ultimately lose their ability to produce hormones but retain the ability to give rise to tumors with restored activity.

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