that reversion is correlated with the partial or complete loss of the RSV-SR genome carried by transformed cells. If this is so, then the reduced transplantability of the reverted cells in the hamster would suggest that the presence of RSV-SR genome is necessary for the expression of those cellular functions which confer the property of increased transplantability. Like polyoma-transformed lines derived from the same cells, the property of transplantability is correlated with altered colonial morphology on glass and the ability to form colonies in agar.

IAN MACPHERSON

Medical Research Council Unit for Experimental Virus Research, Institute of Virology, University of Glasgow, Glasgow, Scotland

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30 April 1965

Phenotypic Alterations in Adrenal Tumor Cultures

Abstract. *Adrenal tumor cells in culture gradually develop a requirement for glutamine and serum albumin in order to produce steroids in response to adrenocorticotropic hormone. Glutamine acts simply as a precursor for a protein or proteins whose synthesis is required for the response to the hormone. Albumin apparently binds steroid hormone products that otherwise would inhibit further biosynthesis of steroids.*

Functional adrenal tumor cultures (*1*) continue to secrete steroids in response to stimulation by adrenocorticotropic hormone (ACTH) at a high rate (4 μg per milligram of protein per hour) for many months in culture. This high rate of steroid synthesis can be maintained in clonal strains which have been in continuous culture for 6 months or more and which have multiplied in culture by a factor of as much as 10^{20} (*2*). Eventually these cultured cells can lose their ability to synthesize steroids, but they retain the ability to give rise to functional tumors (*1*). By the time the cultures have completely lost their capacity to synthesize steroids, they have undergone an extensive loss of adrenal-specific properties (*3*, *4*). Our study is concerned with the early steps of this process before the extensive derangement of cellular properties has occurred.

The ability of adrenal tumor cultures to secrete steroids when incubated for a 2-hour test period with ACTH in simple Krebs Ringer bicarbonate buffer declines with length of time in culture (Fig. 1). Depending on the tumor line, this activity can disappear after 1 week or remain stable for many months in culture. After this activity has declined several fold, the rate of steroid secretion can be made to be as high as the initial rate found immediately after the cells are first cultured, by substituting the tissue culture growth medium for Krebs Ringer buffer as the incubation medium (Fig. 1).

Of the various elements of the tissue culture medium only glutamine and serum albumin increase the biosynthesis of steroid when added to Krebs Ringer buffer. The rate of this synthesis can be as high in buffer to which L-glutamine and albumin have been added as it is in the growth medium (F10 plus dialyzed serum).

The requirement for L-glutamine in this reaction is specific. D-Glutamine, ammonia, glutamic acid, and asparagine are ineffective as substitutes for L-glutamine. The antibiotics 6-diazo-5-oxonorleucine and azaserine do not inhibit the reaction. This lack of inhibition can be taken as strong evidence that glutamine does not act by donating its metabolically active amide group (*5*). Glutamine enhances the biosynthesis of steroids stimulated by adenosine 3′,5′-phosphate, but not the steroidogenesis stimulated by the reduced form of triphosphopyridine nucleotide (TPNH).

Both steroid and protein syntheses, as measured by uptake of ^{14}C-phenylalanine, increase linearly with increasing glutamine concentration, a maximum being reached at 30 μg of glutamine per milliliter (Fig. 2). In view of this coincident dependence on the concentration of glutamine and the lack of inhibition by 6-diazo-5-oxonorleucine and azaserine, the role of glutamine appears to be that of a precursor for protein or proteins whose synthesis is vital for ACTH-induced steroid formation. This necessity for protein synthesis in the activation of steroid synthesis by ACTH has been demonstrated by Ferguson (*6*). Our results indicate that the transfer of phenotypically altered cells from growth medium to incubation buffer results in a depletion of glutamine stores below

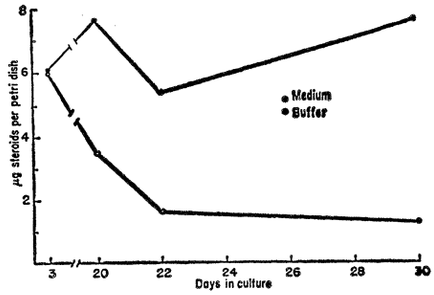

Fig. 1. Adrenal tumor cells were grown in F10 medium containing dialyzed horse serum (10 percent) and dialyzed fetal-calf serum (2.5 percent). At various times after the cultures were initiated they were tested for their capacity to synthesize steroids by incubating them with ACTH for 2 hours in either Krebs Ringer bicarbonate buffer or growth medium.

Table 1. Adrenal tumor cultures were incubated overnight in growth medium containing 0.005 μc of cholesterol-4-^{14}C. The radioactive medium was replaced with Krebs Ringer bicarbonate buffer to which various additions were made. The incubation was carried out for 1 hour more; then steroids were extracted from the buffer and separated by thin-layer silica-gel chromatography. Radioactive material migrating with steroid hormones including pregnenolone was pooled. Radioactive material migrating with cholesterol was assayed separately. The ACTH was added at 5.0 milliunits/ml, pregnenolone at 50 μg/ml, and bovine serum albumin (BSA) at 30 mg/ml.

Additions	Cholesterol (count/min)	Steroids including pregnenolone (count/min)
	Experiment 1	
None	201	411
ACTH	225	2008
ACTH and pregnenolone	254	322
	Experiment 2	
ACTH	323	3458
ACTH and pregnenolone	308	979
ACTH, pregnenolone, and BSA	349	2049

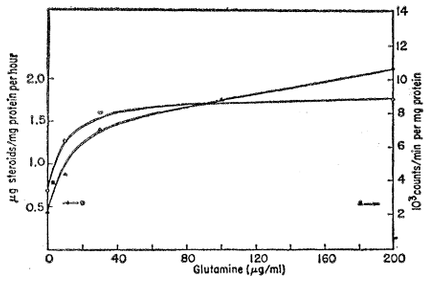

Fig. 2. Cultures were incubated in Krebs Ringer buffer containing 30 mg of bovine serum albumin per milliliter, 5.0 milliunits of ACTH per milliliter, 0.5 μc of ^{14}C-phenylalanine per milliliter, and varying concentrations of glutamine. After 2 hours steroids were extracted from the medium, and the radioactive material (insoluble in hot trichloroacetic acid) incorporated in the cells was determined.

those concentrations necessary to maintain protein synthesis adequate for the ACTH response. Transfer of freshly cultured cells does not result in this depletion of glutamine.

The dependence of steroid formation on the concentration of serum albumin is approximately linear at low concentrations and reaches a maximum at about 30 mg/ml (Fig. 3). When the serum is fractionated by the Cohn method (*7*), the albumin fraction alone is stimulatory. The effect is destroyed by heat and trypsin digestion, but it does not disappear when the albumin is extracted with organic solvents. Thus albumin appears not to act as a reservoir of bound micromolecules. Another way in which albumin could act would be by binding inhibitory substances in

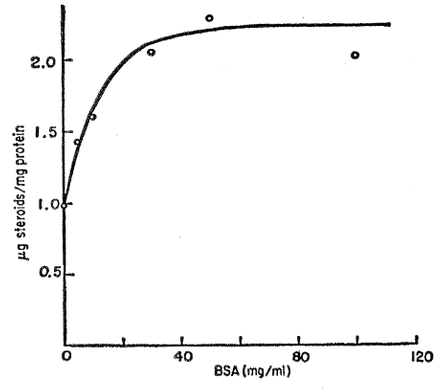

Fig. 3. Adrenal tumor cultures were incubated in Krebs Ringer bicarbonate buffer containing 5.0 milliunits of ACTH per milliliter, 100 μg/ml of glutamine, and varying amounts of bovine serum albumin (*BSA*). After 2 hours the medium was extracted, and its steroid content was determined.

the incubation buffer (Table 1). Adrenal tumor cultures were incubated in growth medium containing cholesterol-^{14}C for 24 hours. The radioactive medium was removed and the cells were incubated in Krebs Ringer buffer containing 30 μg of glutamine per milliliter for 1 hour under various conditions. The medium was harvested and extracted with methylene chloride; the extract was fractionated by thin-layer silica-gel chromatography with a mixture of ether and *N,N*-dimethyl formamide (99 : 1). The radioactive material migrating with the various steroid hormones, including pregnenolone, was eluted and pooled for assay; that migrating with cholesterol was eluted and assayed separately. Adrenocorticotropic hormone stimulates the conversion of radioactive cholesterol, accumulated by the cells in the preincubation period, to steroid hormones. Pregnenolone inhibits the conversion whereas the addition of serum albumin to the incubation buffer partially reverses this inhibition.

Our results represent a technical improvement in the handling of adrenal tumor cultures and raise the possibility that special conditions may be required to demonstrate, in each individual instance, the potentiality of cultured cells to perform differentiated functions. As a further example, epithelium cultures of pigmented iris form melanin only when the growth rate of the cultures is decreased (*8*). Our results provide strong evidence that the loss of specialized function by adrenal tumor cells in culture is actually a change in phenotype in cells that were once functional rather than selective overgrowth by stromal elements of the tumor inoculum. The fact that our cultures lose their ability to synthesize steroids when incubated in Krebs Ringer buffer could be explained on a basis of selective overgrowth, but phenotypic alterations must be invoked to explain the restoration of the synthesizing function by glutamine and albumin.

Finally, these data represent clues in the mystery of phenotypic alterations, an important problem that is receiving more and more attention (*9*).

GORDON H. SATO
TOBY ROSSMAN
LEON EDELSTEIN
STUART HOLMES
VINCENZO BUONASSISI
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

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Hydrogen-Bonded Dimers of Adenine and Uracil Derivatives

Abstract. *In concentrated solutions of either 9-ethyladenine or 1-cyclohexyluracil in deuterochloroform, absorption bands in the infrared spectrum demonstrate hydrogen bonding of the adenine and uracil derivatives with themselves. In dilute solutions, there is very little hydrogen bonding. However, when dilute solutions of 9-ethyladenine and 1-cyclohexyluracil are mixed, a series of bands appear which show that these molecules are hydrogen-bonding with each other much more strongly than with themselves. A study of the stoichiometry of this association indicates formation of 1:1 hydrogen-bonded pairs in solution.*

The molecular basis for the transfer of information in biological systems is believed to be the specificity of hydrogen bonding between the constituent purines and pyrimidines of the nucleic acids. In the double-stranded form of DNA (or RNA), adenine forms hydrogen bonds with thymine (or uracil), and guanine forms such bonds with cytosine. The stability of the two-stranded molecule is governed by hydrophobic or van der Waals interactions and also by electrostatic and hydrogen-bonding interactions. Several studies have been carried out on the interaction of polynucleotides with each other in solution, but no studies have been reported which demonstrate hydrogen bonding between individual pu-