the amount of auxin which can move in the plant is strictly limited by the transport capacity of the tissues (see *3, 12*).

On the other hand, almost all these observations as well as others in the literature (*13*) can be explained as easily on the basis of an "auxin-sparing" action of gibberellic acid (*7, 14*). In fact, Scott and Briggs (*15*) have presented evidence, also based on ether extractions of light-grown "Alaska" peas, that IAA in lanolin added to the 5th internode moves basipetally at about 10 mm/hr for at least 80 mm. Hence, the IAA added in our experiments should have reached the bottoms of the 90 to 100-mm long stems by 10 hours after application. The apparent lack of IAA-C^{14} in the bottom portion of the stem 2 days after the application of IAA alone (Figs. 1 and 2) therefore supports the argument for an effect of gibberellic acid on "auxin-saving" rather than directly on auxin-transport.

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Reversion in Hamster Cells Transformed by Rous Sarcoma Virus

Abstract. *Hamster cells of the BHK-21 line are transformable by Rous sarcoma virus (Schmidt-Ruppin strain). The transformed cells form colonies in agar suspension culture, grow on glass in disarray, and initiate tumors in hamsters and chickens, but extracts do not induce tumors in chickens. Chickens bearing tumors develop neutralizing antibody against the virus. Transformed cell clones give rise to "revertants" which form colonies on glass with cells oriented parallel to each other like the original uninfected cells. These revertants do not grow in agar or initiate chicken tumors, and they regain the original low transplantability of untransformed cells in hamsters.*

When hamster cells of the BHK21 C13 line (*1*) are infected with polyoma virus and suspended in soft agar medium, only cells which have undergone transformation have a high colony-forming capacity. The use of this property in a selective assay for polyoma-transformed hamster cells has been described (*2, 3*). It was of interest to determine whether the same technique was applicable to the study of transformation by other oncogenic viruses. This report describes the use of such technique for isolation of C13 cells transformed by Rous sarcoma virus, Schmidt-Ruppin strain (RSV-SR) (*4*), with particular emphasis on the loss of transformed character by some of these cells.

A suspension of C13 cells was infected with RSV-SR virus at an input multiplicity of 0.05 focus-forming units per cell (*5*). Ten agar suspension cultures were each seeded with 10^4 infected cells. After 14 days at 37°C five colonies about 0.1 to 0.2 mm in diameter and 11 colonies less than 0.1 mm in diameter had developed. Four control plates each containing 10^4 uninfected cells had only a few minute colonies. The five largest colonies from the infected cell cultures were picked and cultured on glass in 10 percent calf serum medium (*1*). The cells in all five cultures grew in disarray like C13 cells transformed by polyoma virus (*1*). When the culture derived from one of these colonies (designated C13/SR) had grown to about 5×10^5 cells, clones were prepared by isolating single cells in microdrops under paraffin (*6*). Three of the 16 isolated cells formed colonies, which were designated C13/SR1, C13/SR2, and C13/SR3. When grown on glass these clones formed randomly oriented cell layers. Cells of the C13/SR2 and C13/SR3 clones grew rapidly and made the medium very acid; C13/SR1 cells grew more slowly. The clones and original C13/SR cells were plated at low cell density to produce discrete colonies in petri dishes containing x-irradiated C13 cells as "feeders" (*7*) (Table 1). A small proportion of the C13/SR, C13/SR2, and C13/SR3 cells formed colonies with well-defined parallel orientation ("revertants"). It is very unlikely that these revertant cells were derived from untransformed C13 cells which had contaminated the transformed colony in the agar culture and which had been carried through the cloning procedure. The micromanipulations of cloning involve two separate visual checks on the discreteness of the isolated cell. The possibility that the revertants were derived from the x-irradiated feeder cells was excluded when platings of C13/SR3 cells with and without feeder cells gave essentially the same proportion of transformed and reverted colonies. Clone C13/SR1 cells gave rise to only transformed colonies, that is, with random orientation. Intermediate colonies with some degree of parallel orientation in the center of the colony were present in all platings except those of C13/SR1 cells.

This gradation in morphology made classification of the colonies difficult, but in the results presented here only colonies with very well-marked parallel orientation, like the original C13 cells, have been classified as "reverted." A completely random transformed colony and a revertant colony in a plating of C13/SR3 cells are shown in Fig. 1. When platings were made in fetal instead of postnatal calf serum the proportion of transformed colonies was increased, but well-marked reverted colonies were still present in platings of C13/SR2 and C13/SR3 (Table 2). This observation is of particular interest since Rubin (*8*) has shown that

Table 1. Colony formation by C13/SR cells and its derivative clones in early passage in 10 percent serum medium.

Cells	Plating efficiency (%)	Colonies with parallel orientation (revertants) (%)
C13/SR	35	10
C13/SR1	21	0
C13/SR2	55	1.3
C13/SR3	55	9.4

Table 2. Transplantation of C13/SR clones and subclones in hamsters and chickens.

Percentage of transformed colonies in platings at times of transplantation in:		No. of tumors and No. of animals inoculated			
		Hamsters *			Chicken: †
10% calf serum	10% fetal calf serum	Dose 10^6 cells	Dose 10^4 cells	Dose 10^2 cells	Dose 5×10^6 cells
		C13/SR2 (transformed)‡			
94	94	8/8	7/8	5/8	2/4
		C13/SR3/2a (transformed)§			
25	99	6/8	6/8	1/8	3/4
		C13/SR3/2f (transformed)§			
22	98	7/8	5/8	0/8	1/4
		C13/SR3/11f (reverted)¶			
0	0	4/8	1/8	0/8	0/4
		C13/SR3/15h (reverted)¶			
0	0	2/8	0/8	0/8	0/4
		C13			
0	0	3/16	0/8		
		C13–TC6			
100	100	5/6	8/8	7/8	

* Cheek-pouch tumors, after 30 days. † Right breast muscle. ‡ C13/SR2 and C13/SR3 are transformed clones of C13/SR cells. § C13/SR3/2a and C13/SR3/2f are subclones of C13/SR3 which were transformed when first isolated. ¶ C13/SR3/11f and C13/SR3/15h are subclones of C13/SR3 which were reverted when first isolated.

fetal calf serum inhibits the formation of transformed foci by Rous virus in chick-embryo cultures.

Cells of the C13/SR3 clone were recloned in microdrops. Single transformed and reverted colonies from platings of C13/SR3 cells in postnatal calf serum medium without feeders provided the cells for these isolations. Three transformed subclones and 16 revertant subclones were isolated in this way. The three original clones and some of the subclones were carried in serial subculture and were plated on feeders and in agar suspension culture at various passages.

Clone C13/SR1 cells remained 100 percent transformed, but after two passages the cells degenerated and were lost. Clone C13/SR2 soon attained a stable distribution of colonial forms. In seven platings over a period of 7 weeks, about 6 percent of the colonies in postnatal calf serum were revertants. Colony-forming efficiency in agar was from 1 to 2 percent during this period.

In the case of C13/SR3 the proportion of revertant colonies increased from 5.5 percent to 19 percent after 3 weeks and eventually to 98 percent after 8 weeks. A similar progression was observed in two subclones which were originally transformed, namely, C13/SR3/2a and C13/SR3/2f. In agar suspension culture a progressive drop in plating efficiency from 4 percent to less than 0.1 percent also occurred with passage. Platings of four revertant subclones of C13/SR3 cells, which had been carried in serial subculture in postnatal calf serum medium, gave rise to colonies with exclusively parallel orientation. Three of these clones were also completely reverted in fetal calf serum medium, but the fourth contained 11 percent transformed colonies. In agar culture none of the subclones produced more than 1 or 2 colonies per 10^4 cells plated.

Transplantations of C13/SR clones and subclones were made intradermally in the cheek pouches of young adult hamsters. The results in Table 2 show that the very low transplantability of the original C13 cells is greatly increased by RSV-SR transformation and that the revertant cells have lost this increased transplantability. The results of transplanting a polyoma-transformed derivative of C13, namely, C13-TC6, in the hamster cheek pouch are also included in Table 2.

In order to test the virus content of the transformed cells eight commercial white Leghorn chickens were inoculated with 5×10^6 C13/SR cells in the right breast muscle and with an extract (frozen and thawed three times) of 4×10^7 cells in the left breast muscle. At autopsy within 3 weeks it was found that firm walnut-sized sarcomas had developed in the right breast muscles. The serums from four birds with tumors were tested for neutralizing activity against RSV-SR virus (*5*); all four were positive. Cultures of sarcoma tissue from two birds tested were identified karyologically as chicken cells, but no cells with hamster chromosomes were found. Similar results were obtained when clones containing predominantly transformed cells were inoculated into chickens. The reverted clones tested so far have failed to induce tumors in chickens (Table 2). Thin sections of C13/SR cells have been examined by electron microscopy and no virus-like particles have been detected.

Although the details of the cell–virus relationship in this system remain to be worked out, it is possible

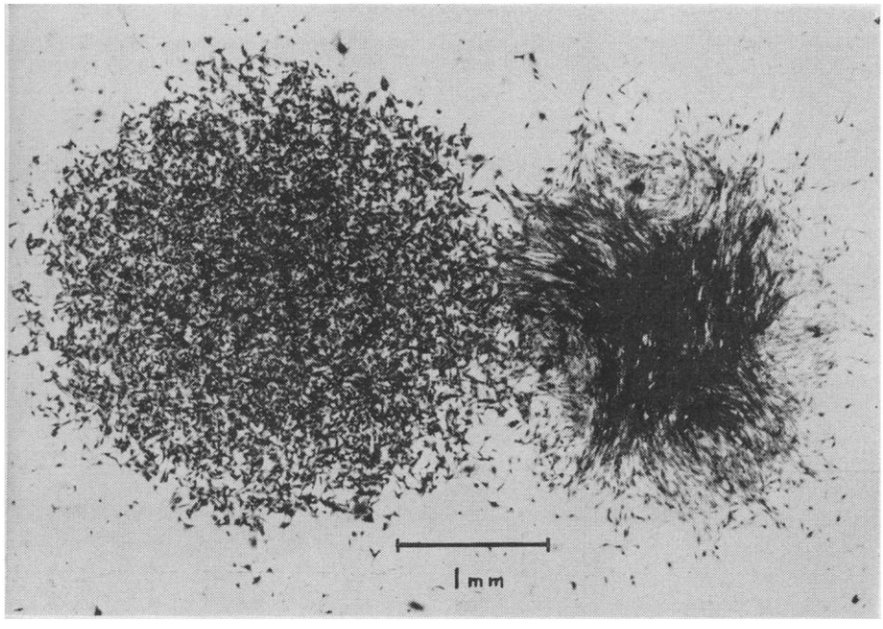

Fig. 1. A transformed colony (left) and a revertant colony (right) from an early plating of C13/SR3 cells cultured for 7 days in 10 percent calf serum medium.

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.

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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

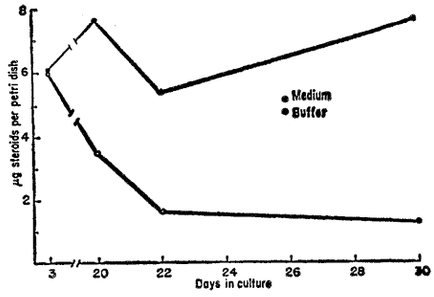

Fig. 1. Adrenal tumor cells were grown in F10 medium containing dialyzed horse serum (10 percent) and dialyzed fetal-calf serum (2.5 percent). At various times after the cultures were initiated they were tested for their capacity to synthesize steroids by incubating them with ACTH for 2 hours in either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