yields from pooled parotid saliva. Fourth, parotid saliva samples of the three phenotypes, as determined by the 3,3′-dimethoxybenzidine stain on polyacrylamide gel, were subjected to fractionation on a Sephadex G-50 column. The individual fractions were monitored by disc-gel electrophoresis. In the elution profile of the two homozygous samples, only proline-rich proteins I and III or II and IV could be detected, whereas, in the case of the heterozygous sample, all four proline-rich proteins were detected (Fig. 2, top). Finally, the electrophoretic mobility of the four purified, proline-rich proteins was identical with the unpurified salivary proteins exhibiting genetic polymorphism on slab polyacrylamide-gel electrophoresis (Fig. 2, bottom). These data make it highly likely that the proteins showing polymorphism are identical to the proline-rich proteins previously purified and characterized (*1*).

These proline-rich proteins were also found in submaxillary fluid from adults as well as in the parotid saliva of newborns and premature infants. Interconversion, or disappearance, of the proline-rich proteins was not seen after incubation of the saliva at 37°C for up to 2 hours. Furthermore, patterns of proline-rich proteins were easy to type and were stable after storage of samples for at least 9 months at −70°C. Initial data (*6*) from linkage studies indicate that there is no correspondence between the proline-rich protein phenotypes and the phenotypes observed in the following genetic systems: ABO, Rh, MNSs, Kell, Kidd, Lutheran, Duffy, Lewis, secretor, haptoglobin, transferrin, group-specific components, parotid basic protein, and hemoglobin (beta chain determinant).

The parotid saliva contains many interesting proteins, only a few of which have been isolated and characterized. It is also an easily obtained source of secretory proteins and has proved useful in the search for new genetic polymorphisms, as indicated by the proline-rich (Pr) protein polymorphism herein described and the recently reported parotid basic (Pb) protein polymorphism in man (*5*).

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4. Parotid fluid samples were collected from adults, children, and infants with the use of an acrylic capsule and hard lemon candy stimulation (*5*): Samples were routinely stored at −70°C. A standard alkaline polyacrylamide-gel slab was prepared by mixing 200 ml of tris borate–boric acid buffer, *p*H 8.6 to 8.9 (0.088*M* in tris), 11.0 g of Cyanogum (E-C Apparatus Corp., Philadelphia), 0.2 ml of *N, N, N′,N′*-tetramethylethylenediamine, and 2.0 ml of 10 percent ammonium persulfate. Gels were cast in a vertical electrophoresis apparatus with the use of a special Teflon slot former (E-C Apparatus Corp.). The bridge solution was of the same composition as the gel buffer. Prior to electrophoresis, frozen samples were lyophilized and dissolved in tris borate–boric acid gel buffer to concentrate the samples about five to ten times. Sucrose (15 mg/0.1 ml) and bromphenol blue as a marker were also added. The samples were centrifuged at 35,000*g* for 15 minutes and were electrophoresed in the polyacrylamide gels for approximately 3½ hours at constant voltage (250 volts) with tap water (12° to 15°C) cooling, after a preliminary run of about 2 hours. The gels were then stained at room temperature for about 30 to 60 minutes with a solution containing 400 to 600 mg of 3,3′-dimethoxybenzidine (Eastman) and 5.0 ml of glacial acetic acid per 100 ml. The resulting patterns could be seen as translucent gray-blue bands against a gray, somewhat opaque background when viewed by reflected light against a black background. The patterns could be typed and photographed, but the background could be advantageously darkened by addition of hydrogen peroxide. The staining solution was then poured off and fresh staining solution was added to which 0.1 to 0.3 ml of hydrogen peroxide per 100 ml of solution was added. The solution turned brown, and after about 30 to 60 minutes at room temperature, a pattern of white bands could be seen against a brown background by transmitted light. Depending on the lot used, the concentration of 3,3′-dimethoxybenzidine and hydrogen peroxide may need to be varied to produce the proper brown color for rapid staining. To remove a superficial brown deposit on the gel after staining, the surface was rinsed with water and gently rubbed by a hand covered with a latex glove. The phenotypes were easy to visualize, although they were more difficult to photograph. When the patterns could not be seen because of inadvertent overstaining, the gel could be partially destained with visualization of the patterns within several hours after washing in 2 percent ascorbic acid. Although the reason for negative staining reaction of the proline-rich proteins is not entirely clear, it appears that when the gel is initially exposed to acidified 3,3′-dimethoxybenzidine, the 3,3′-dimethoxybenzidine precipitates in the gel, but this precipitation is inhibited at the positions of the proline-rich proteins, which appear as translucent bands against an opaque background. In the next step, after addition of fresh stain solution containing hydrogen peroxide, the opaque background takes a brown stain and the proline-rich proteins appear as white bands by transmitted light.
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7. Supported by NIH grants AMO 9555-07, DEO 3658-08, and DEO 3433-01. We thank Mrs. H. Lin for technical assistance, Dr. W. Bias for doing genetic typing and providing saliva samples, and Dr. O. Smithies for helpful discussions.

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Virus-Induced Transformation without Cell Division

Abstract. *Interference with the cell cycle by vinblastine sulfate immediately after cells were infected with Rous sarcoma virus had little effect on the development of two metabolic changes that occur in transformed cells. These results, along with an earlier demonstration of morphological changes developing in infected nondividing cells, demonstrate that the phenotypic development of the malignant state can occur without the intervention of cell divisions after infection by Rous sarcoma virus.*

Transformation of cells to malignant forms commonly is observed several cell generations after the initial carcinogenic event. The assessment of transformation in cell culture is based on morphological changes in the cells (such as increased rounding or refractility), on altered associations between cells (for instance, more random orientation or increased cell density at growth saturation), or on altered growth potential (such as increased growth in suspension). Although the malignant change can occur in the single cell, the identification of malignant transformation usually has required an accumulation of transformed cells several cell generations after induction. Transformation by Rous sarcoma virus (RSV), particularly the Bryan "high titer" strain, has the unique advantage that single transformed cells can be identified by their characteristic morphology. The ability to identify individual transformed cells allowed us to show earlier that a single round of DNA synthesis was sufficient for successful transformation by RSV (*1*), and that morphological transformation can occur in RSV-infected cells that have not divided (*2*).

Morphological changes may be considered an insufficient criterion for malignant transformation, and are difficult to assess quantitatively. However, biochemical changes often accompany malignant transformation, and these can be used as a measure of transformation. Cells transformed by RSV have an increased capacity for hexose

Table 1. Uptake of 2-[^{3}H]deoxyglucose by RSV-infected cells treated with vinblastine. Chick embryo primary cultures that were confluent and nondividing were treated with trypsin and replated. Cells were allowed to settle for 6 hours, during which time no mitoses had yet occurred. The cells were then exposed to RSV (approximately 10 focus-forming units per cell), and vinblastine sulfate (0.1 μg/ml) in growth medium (*5*) was added. Cultures were maintained at 39°C with daily changes of vinblastine-containing medium. At the indicated times, culture fluids were removed and 2-[^{3}H]deoxyglucose (1 μc per plate) in Dulbecco's phosphate-buffered saline (PBS) was added. After a 15-minute incubation at 39°C, the cultures were gently rinsed four times with cold PBS, 0.5 percent Nonidet P-40 in PBS was added, and radioactivity and protein were determined.

Treatment	Radioactivity (count/min per microgram of protein)	
	Noninfected	RSV-infected
16 hours after infection		
None	353	626
Vinblastine	153	187
40 hours after infection		
None	403	954
Vinblastine	163	326
64 hours after infection		
Vinblastine	323	944

uptake (*3*) and synthesize more hyaluronic acid than do noninfected cells or cells infected with nontransforming avian leukosis viruses (*4, 5*). The following study shows that these biochemical characteristics of transformed cells can be found in cells that have never divided after infection with RSV.

Vinblastine sulfate is commonly used in cell culture systems to accumulate cells in mitosis for chromosome analyses. Earlier experiments (*2*) showed that cultured chick embryo cells failed to divide when exposed to vinblastine. Cells were replated from nondividing confluent primary cultures, and after 6 hours, before any mitoses were evident, cells were exposed to vinblastine. No increase in cell number was seen after this time in cultures treated with vinblastine concentrations above 0.015 μg/ml (Fig. 1). About two-thirds of the treated cells became rounded in the course of an 18-hour exposure to vinblastine (0.1 μg/ml), but these rounded cells could be distinguished from normal mitotic cells by the absence of microscopically visible metaphase chromosomes. Also, no recently divided cells or cells in late anaphase or telophase were seen in cultures containing vinblastine. The remaining third of the cells stayed adherent to the substratum and assumed epithelioid shapes; in these cells morphological changes characteristic of viral transformation have been observed after infection by RSV (*2*).

The uptake of hexose into RSV-infected and uninfected cells, with or without vinblastine (0.1 μg/ml), was examined. Within 16 hours after infection, infected untreated cells showed increased capacity to incorporate 2-[^{3}H]-deoxyglucose compared to uninfected controls, and by 40 hours after infection the rate of incorporation into infected cells was more than double that of uninfected cells (Table 1). These results are similar to those previously described (*3, 5*).

Likewise, infected cells that were treated with vinblastine and never reached mitosis showed increased capacity to incorporate 2-[^{3}H]deoxyglucose compared to uninfected cells. Although observable differences were minimal 16 hours after infection, by the third day infected vinblastine-treated cells incorporated 2-deoxyglucose at nearly three times the rate of uninfected cells. The lower values of vinblastine-treated cultures compared to untreated cultures at 16 and 40 hours can be attributed partially to residual rounded (mitotic) cells, which have a decreased potential for 2-deoxyglucose uptake; these cells became detached during the course of the experiment and were removed by rinsing.

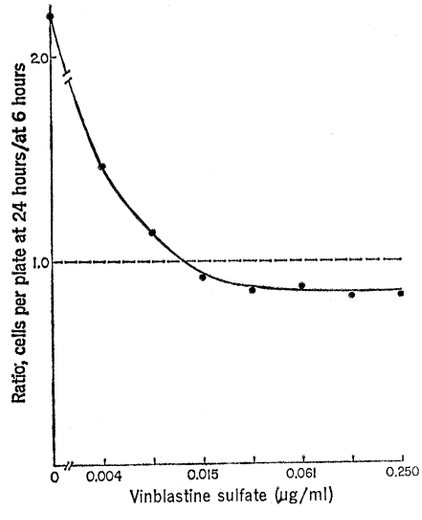

Fig. 1. Effect of vinblastine on cell division. Confluent chick embryo primary cultures were treated with trypsin to release cells and replated (about 7×10^6 cells per 10-cm petri dish). Six hours later the medium containing floating cells was removed from all cultures, and adherent cells were released with trypsin and counted from sample cultures. Vinblastine sulfate in growth medium was added to the remaining cultures, and 18 hours later rounded floating cells and adherent cells released by trypsin were pooled and counted. No dividing cells or cells in late anaphase or telophase were observed microscopically.

Table 2. Production of hyaluronic acid by RSV-infected cells treated with vinblastine. Cultures were infected and treated as in Table 1. Fluids were removed from cultures at indicated times, centrifuged to remove cellular debris, exposed to Pronase (50 μg/ml) at 37°C for 30 minutes, made 0.1*N* in NaOH, dialyzed for 72 hours against water, and lyophilized to dryness. The dried material was reconstituted in one-fifth the original volume, and portions were analyzed by electrophoresis in polyacrylamide-agarose mixed gels as described (*6*).

Treatment	Hyaluronic acid (micrograms per milligram of protein)	
	Noninfected	RSV-infected
16 hours after infection		
None	7.2	14.0
Vinblastine	3.5	8.0
40 hours after infection		
None	8.1	38.0
Vinblastine	2.9	12.9

Synthesis of hyaluronic acid also increased in chick embryo cells after infection with RSV (Table 2). As in the case of 2-deoxyglucose uptake, treatment with vinblastine did not prevent an increase in hyaluronic acid in infected compared to uninfected cultures, although the amounts of hyaluronate per milligram of cell protein were not as high in vinblastine-treated cultures as in their nontreated counterparts.

In these as in previous studies, cells exposed to vinblastine failed to divide; in fact, many never reached mitosis. The results demonstrate that two biochemical changes, which characteristically occur during transformation of cells by RSV, occur in newly infected cells without an intervening cell division. Studies on cells infected with a mutant of RSV demonstrated that increased hexose uptake and hyaluronic acid synthesis are both dependent on new RNA and protein synthesis (*5*). It is apparent then that a battery of metabolic changes have occurred in these cells, and the observed changes cannot be attributed merely to structural changes that might result from virion attachments to the cell surface membrane.

Earlier studies demonstrated that RSV can proceed through a full cycle of reproduction in vinblastine-treated cells, and that cells prevented from dividing after infection can become morphologically transformed (*2*). These combined studies show that cell division probably is not a prerequisite for the phenotypic expression of malignancy; that is, RSV-infected cells prob-

ably have the potential for malignancy irrespective of cell division, although malignancy cannot be demonstrated without cell divisions. While it may be unreasonable to extrapolate to other systems of carcinogenesis, one may wonder whether cell divisions, per se, are ever required for the phenotypic development of the malignant state.

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Estimation of the Half-Life of a Secretory Protein Message

Abstract. *Synthesis of a specific secretory protein was followed in the presence of actinomycin D and cordycepin (3′-deoxyadenosine). The apparent half-life of the messenger RNA for this protein is significantly longer in the presence of actinomycin D than in the presence of cordycepin. These results suggest that actinomycin D studies of half-life in specialized cells may be inaccurate.*

Various workers have used actinomycin D to study the half-life of specific messenger RNA (mRNA) and concluded that many highly differentiated eukaryotic cells are characterized by long-lived mRNA molecules (*1*, *2*). An excellent system for such studies is provided by the large parenchymal cells of the colleterial gland tubules of the giant female saturniid moth *Hyalophora cecropia*. The secretory protein, synthesized and transported by these parenchymal cells, incorporates at least 90 percent of the total [^{3}H]-glycine label fixed during a measured short period in vitro (Fig. 1). Thus the incorporation of the glycine label into the acid-insoluble fraction serves as a reliable estimate of the amount of secretory protein synthesized.

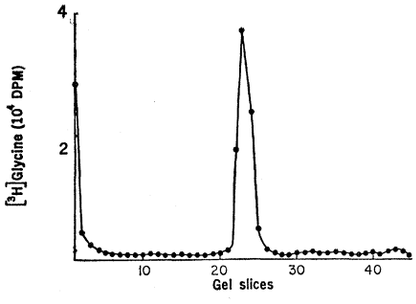

Fig. 1. Electrophoretic separation of colleterial gland proteins. Colleterial tubules from *H. cecropia* at "day 16 to 17" of development (*14*) were labeled for 4 hours in vitro with [^{3}H]glycine (4.67 μc/ml; 10.7 c/mmole; New England Nuclear). The tubules were washed and homogenized in insect Ringer solution and subjected to electrophoreses on 7.5 percent polyacrylamide disc gels according to the methods of Davis (*15*). Approximately 90 percent of the total [^{3}H]glycine entering the gel was found in a single peak when the material was migrating toward the anode. Gels were sliced transversely into 1-mm sections and dissolved in 0.3 ml of 30 percent H_2O_2 before the addition of a toluene-based counting fluid containing 10 percent Biosolve BBS–3 (Beckman). DPM, disintegrations per minute.

In an attempt to study the half-life of the specific mRNA for this glycine-rich protein, we used actinomycin D to inhibit new mRNA synthesis according to the methods of Kafatos and Reich (*1*). Protein synthesis as measured by incorporation of [^{3}H]glycine at various times after actinomycin D inhibition continued at a substantial rate. This result appeared to confirm results associating a specific long-lived mRNA molecule with a highly differentiated cell (*1*, *2*).

Our results (Fig. 2) indicate that, after treatment with actinomycin D at twice the concentration necessary to inhibit 95 percent incorporation of [^{3}H]uridine, incorporation of labeled glycine into protein continues at 70 percent of the control value for at least 12 hours. This 70 percent incorporation is the lowest we observed, and in fact we sometimes observed in the treated tissue a slight stimulation of protein synthesis over that in controls. The mRNA half-life calculated on the basis of the experiments in Fig. 2 would be considerably longer than 12 hours. Further experiments demonstrated that, although the amount of stimulation of protein synthesis was not consistent in the presence of actinomycin D, the rate of protein synthesis compared to controls remained relatively high for at least 24 hours. The occasional stimulation of protein synthesis observed in several experiments during treatment with actinomycin D suggested the possibility of an interaction between actinomycin D and mRNA. Singer and Penman (*3*) have suggested that actinomycin D has a stabilizing effect on mRNA in HeLa cells and concluded that actinomycin D studies on mRNA half-life may not always be correct. We undertook further experiments comparing the effects of actinomycin D and cordycepin, another inhibitor of RNA synthesis, on the half-life of mRNA.

Cordycepin (3′-deoxyadenosine), an adenosine analog, is similar to actinomycin D in that it has selective inhibitory properties (*4*, *5*). Cordycepin suppresses the labeling of cytoplasmic mRNA (*3*) and may function in part by blocking transport of mRNA from the nucleus (*6*, *7*). Labeling of intact colleterial tubules with [^{3}H]glycine at various times during treatment with cordycepin produced striking differences in the estimated half-life of the specific mRNA when compared to results obtained with actinomycin D. Protein synthesis was suppressed by 90 percent 4 hours after prior treatment with cordycepin, and the mRNA half-life was approximately 2 hours. This rapid suppression of protein synthesis in the presence of cordycepin suggested the possibility that actinomycin D might somehow stabilize mRNA. Results of experiments with cordycepin and actinomycin D in combination produced a pattern of inhibition con-

Table 1. The tubules of a mature colleterial gland of *H. cecropia* were treated with [^{3}H]-uridine (25 μc/ml; 25.7 c/mmole; New England Nuclear) in Grace's insect T.C. medium at 25°C for 10 minutes and then washed twice with fresh medium. Pieces of the tubules were then treated with either medium alone or medium supplemented with actinomycin D (25 μg/ml) or cordycepin (100 μg/ml). At 5 hours from the start of treatment, the tissue was washed in fresh medium and fixed in Bouin's solution. Sections (5 μm) were treated twice with 5 percent trichloroacetic acid for 5 minutes each and then prepared for autoradiography. Silver grains were counted over random 2000 μm^2 of tissue for both the basal half of the secretory cells (N) which contains the nucleus and the apical half (C) which contains most of the protein synthesizing machinery. The N/C ratio is an indication of the relative concentration of labeled RNA in nucleus and cytoplasm.

Treatment	Silver grains		N/C
	N	C	
Control	5074	1503	3.37
Actinomycin D	960	519	1.85
Cordycepin	1848	446	4.14