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.

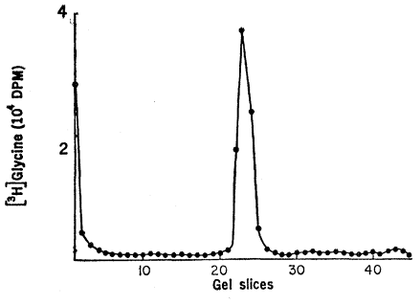

Fig. 1. Electrophoretic separation of colleterial gland proteins. Colleterial tubules from *H. cecropia* at "day 16 to 17" of development (*14*) were labeled for 4 hours in vitro with [^{3}H]glycine (4.67 μc/ml; 10.7 c/mmole; New England Nuclear). The tubules were washed and homogenized in insect Ringer 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

sistent with that for cordycepin alone, indicating either that no stabilization of mRNA took place or that cordycepin has an alternative effect which counteracts the results of stabilization. Butcher *et al.* (*8*) found that cordycepin, in contrast to actinomycin D, does not stimulate specific protein synthesis in hepatoma cells.

Interference with energy transfer could explain the rapid decline in protein synthesis observed during cordycepin treatment. Plagemann (*5*) has shown that cordycepin had no effect on the phosphorylation of adenosine to the triphosphate in hepatoma cells, indicating that the drug does not uncouple oxidative phosphorylation. Using Warburg respirometers (*9*) we made an analysis of oxygen consumption on whole colleterial tubules in the presence of actinomycin D or cordycepin. The respiration of tubules (measured as milliliters of O_2 consumed per gram per hour) was comparable to that of controls at 2 hours from the beginning of inhibitor treatment; however, at 4 hours, oxygen consumption had been suppressed 50 percent by actinomycin D and only 33 percent by cordycepin. These results support our belief that cordycepin has no greater effect on energy transfer than does actinomycin D. Butcher *et al.* (*8*) compared the effects of actinomycin D and cordycepin on the "superinduction" of tyrosine aminotransferase in hepatoma cells and have found that cordycepin does not interfere with mRNA translation.

The striking difference observed in the apparent mRNA half-life during actinomycin D or cordycepin treatment may be explained by examination of reported data on cordycepin inhibition. Evidence that cytoplasmic mRNA is derived from giant heterogeneous nuclear RNA (HnRNA) has been based, in large part, on the appearance of similar-sized polyriboadenylic acid [poly(A)] sequences in both species of RNA (*10, 11*). The mechanisms by which poly(A) becomes associated with HnRNA or mRNA and by which HnRNA serves as precursor to mRNA are still not clear (*6, 7, 10*). In any event, cordycepin apparently blocks poly(A) synthesis, mRNA biogenesis, and possibly transport of mRNA to the cytoplasm (*6, 7, 12*). The fact that protein synthesis continues for a prolonged period in the presence of an actinomycin D dosage sufficient to inhibit virtually all RNA synthesis, but not in the presence of cordycepin, supports the possibility that cordycepin, by inhibiting poly(A) metabolism, interferes with mRNA transport from nucleus to cytoplasm. A large highly polyploid nucleus such as that found in the colleterial parenchymal cells could serve as a storage depot for either mRNA or mRNA precursor molecules (HnRNA) or both. Adesnick *et al.* (*12*) concluded that potential mRNA molecules remain in HeLa cell nuclei for considerable lengths of time after poly(A) addition is completed. Treatment with actinomycin D which inhibits total RNA synthesis may not affect poly(A) synthesis equally (*6*); therefore, a continued "processing" of mRNA from precursor, with the addition of poly(A), could take place in the presence of actinomycin D. Slater *et al.* (*13*) have demonstrated that actinomycin D is totally ineffectual in inhibiting poly(A) synthesis in developing sea urchin embryos. Cordycepin, however, partially suppresses poly(A) synthesis and induces an irreversible developmental block. We have shown autoradiographically that cordycepin suppresses the transport of RNA from the nucleus, whereas actinomycin D does not (Table 1).

We conclude that, by virtue of cordycepin's inhibition of poly(A) synthesis and disruption of mRNA processing from precursor molecules or by some other mechanism which blocks mRNA transport, the shorter half-life of mRNA determined from cordycepin experiments may be more accurate than that observed in the presence of actinomycin D. We propose that one possibility for the longer half-life observed in the presence of actinomycin D may be related to the replenishment of cytoplasmic mRNA from a large nuclear pool.

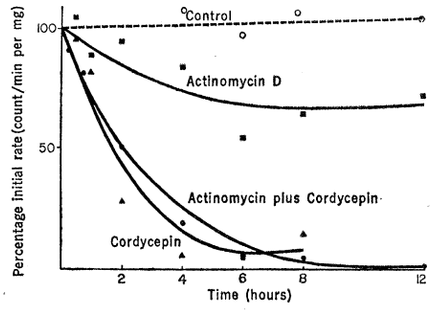

Fig. 2. Rates of protein synthesis in cultured colleterial parenchymal cells. Pieces of colleterial tubules from *H. cecropia* pupae dissected at days 16 to 17 of adult development in Grace's insect T.C. medium (Grand Island Biological) were first treated for 2 hours with either actinomycin D (25 μg/ml) or cordycepin (100 μg/ml), or a combination of the two inhibitors. (Rates of incorporation varied considerably for the first 2 hours of actinomycin D treatment but became stabilized thereafter.) Controls were maintained in Grace's medium without inhibitors. After the preliminary treatment tissue was labeled for 15 minutes with [^{3}H]-glycine (4.67 μc/ml; 10.7 c/mmole; New England Nuclear) at various times (0 to 12 hours) during the ensuing inhibitor treatment. After being washed in Grace's medium, the tissue was homogenized with insect Ringer solution in a ground-glass homogenizer and centrifuged for a short time in a clinical centrifuge. Protein concentrations were determined by the method of Lowry (*16*). The remaining supernatant was treated with cold 5 percent trichloroacetic acid and the insoluble material was collected on glass Millipore filters and analyzed in a scintillation spectrometer (Chicago Nuclear Mark I). Incorporation is expressed as a percentage of the value obtained immediately after the 2-hour preliminary treatment with inhibitor. Initial values were all between 4,000 and 11,000 count/min per milligram of protein.

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