physicochemical environment that can substitute for the intraphagosomal milieu seems fundamental to further successes. The Nakamura system is an example of the serendipity that arises in basic research. It seems unlikely that seven key factors—oxygen restriction, the presence of sulfhydryl groups that meet the specific sulfur requirements of *M. lepraemurium*, and five supplements—could have been chosen and aligned without the guidelines provided by the phenomenon of elongation. Nakamura's choice of organism was fortunate, because *M. leprae* and other noncultivated organisms do not afford morphologic evidence of active systems in vitro. For such organisms, biochemical indicators of physiologic status will be needed to measure the effects of favorable factors in the absence of growth. Metabolic methods such as respiration and hydrogen transfer capacity (*6*) proved of limited use because of the leakiness of intracellular microorganisms. By the time such organisms have been washed free of host components, they retain only vestiges of their normal metabolic activity (*7*). A merit of measuring ATP is that data can be obtained immediately from unwashed host-grown organisms. Such data have here been shown to indicate (i) the lowest point of energy production by type 1 (host-adapted cells); (ii) the point at which ATP production again increases, which shows that type 2 cells have become functional; (iii) the time required for the organism to produce energy in vitro in the same quantities as in vivo; and (iv) the expansion of cell populations thereafter.

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8. This work was supported in part by grants from the National Institutes of Health (AI-8866), World Health Organization, and Damien-Dutton Society, New York. We thank H. B. Funk for assistance in abbreviating the original manuscript.

10 January 1977; revised 4 March 1977

Stimulation of in vitro Translation of Messenger RNA by Actinomycin D and Cordycepin

Abstract. *Actinomycin D and cordycepin were tested for their effect on translation in the wheat germ embryo extract and reticulocyte lysate assays for in vitro protein synthesis. Both drugs were found to stimulate the incorporation of ^{35}S-labeled methionine into protein up to threefold as compared to control assays. This was true for synthesis directed by murine myeloma polyadenylate–containing RNA, tobacco mosaic virus RNA, and endogenous reticulocyte messenger RNA.*

Actinomycin D and cordycepin (3′-deoxyadenosine) are inhibitors of transcription that have been widely used in studies of gene regulation. In order to eliminate ambiguity in interpreting the results of several kinds of experiments—for example, determinations of the half-life of messenger RNA (mRNA) (*1*) and superinduction studies (*2*)—it is necessary to assume that these compounds do not significantly affect translation. However, this assumption has been tested for actinomycin D with conflicting results. Revel and Hiatt (*3*) observed as much capacity for protein synthesis in rat livers exposed to actinomycin D for 40 hours as in control livers. Palmiter and Schimke (*4*) analyzed the effect of actinomycin D on ovalbumin mRNA translation and found an increase in the rate of in vivo elongation with a maintenance of polysome size. These results imply an increase in the rate of polypeptide initiation and thus in translation in the presence of actinomycin D. Another study (*5*) showed a contrary result, an actinomycin D–induced decay in the rate of protein synthesis in HeLa cells which was attributed to an effect on the association of mRNA with ribosomes. Sargent and Raff (*6*) investigated the effect of actinomycin D on protein synthesis and polyadenylation capacities of mRNA in activated enucleate sea urchin eggs and found no effect of the drug on either process; findings in the sea urchin system may not be applicable to other systems because of the conditions of the mRNA stability and the permeability of sea urchin eggs. A problem common to all but the last study listed above is that it is nearly impossible to uncouple transcription and translation in intact cells.

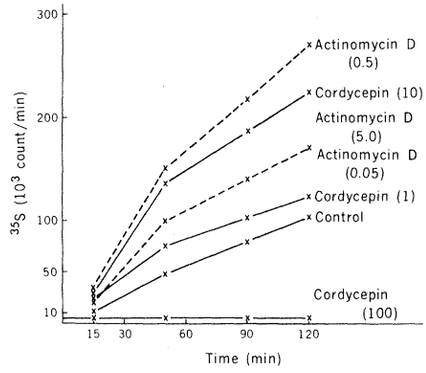

Fig. 1. Incorporation of ^{35}S-labeled methionine into protein by myeloma mRNA in wheat germ embryo extracts as described below. Actinomycin D and cordycepin were added in 1-μl volumes (at concentrations indicated) to 25-μl assays to which was added 1 μg of myeloma poly(A)–containing RNA. Portions (5 μl) were removed at indicated times, and incorporation of isotope into material precipitable by trichloroacetic acid was determined. Myeloma mRNA was isolated from RPC5.4 cells by proteinase K (EM Laboratories) digestion of supernatants from which the mitochondria had been removed (*9*); the supernatants were then chromatographed on oligo(dT) cellulose (*10*). The RNA was translated in a wheat germ extract system as described (*11*), except for the following modifications: 28 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), *p*H 7.1, in place of tris; 40 μ*M* spermine was included in the assay mixture; 80 m*M* K^+ in place of 120 m*M* K^+. Incubations were at room temperature.

Our studies were designed to answer two questions: (i) Do transcriptional inhibitors such as actinomycin D and cordycepin affect in vitro translation? And, if so, (ii) are the protein products affected differentially?

We have studied directly the effect of actinomycin D and cordycepin on protein synthesis by using in vitro translation systems. Two types of extracts have been used to investigate the effect of the drugs on protein synthesis: the wheat germ embryo extract system and the reticulocyte lysate system. The wheat germ system was chosen because it has very low endogenous incorporation and is very highly stimulated by many prokaryotic and eukaryotic mRNA's. We have used mouse myeloma RNA that contains polyadenylate [poly(A)] and RNA from tobacco mosaic virus (TMV) to direct synthesis in wheat germ assays and used translation of endogenous mRNA's in reticulocyte lysates. Our experiments demonstrate that actinomycin D and cordycepin stimulate in vitro incorporation of ^{35}S-labeled methionine into polypeptides directed by TMV, myeloma, and endogenous reticulocyte mRNA up to threefold as compared to the controls.

Each drug increased both the rate and extent of incorporation of [^{35}S]methi-

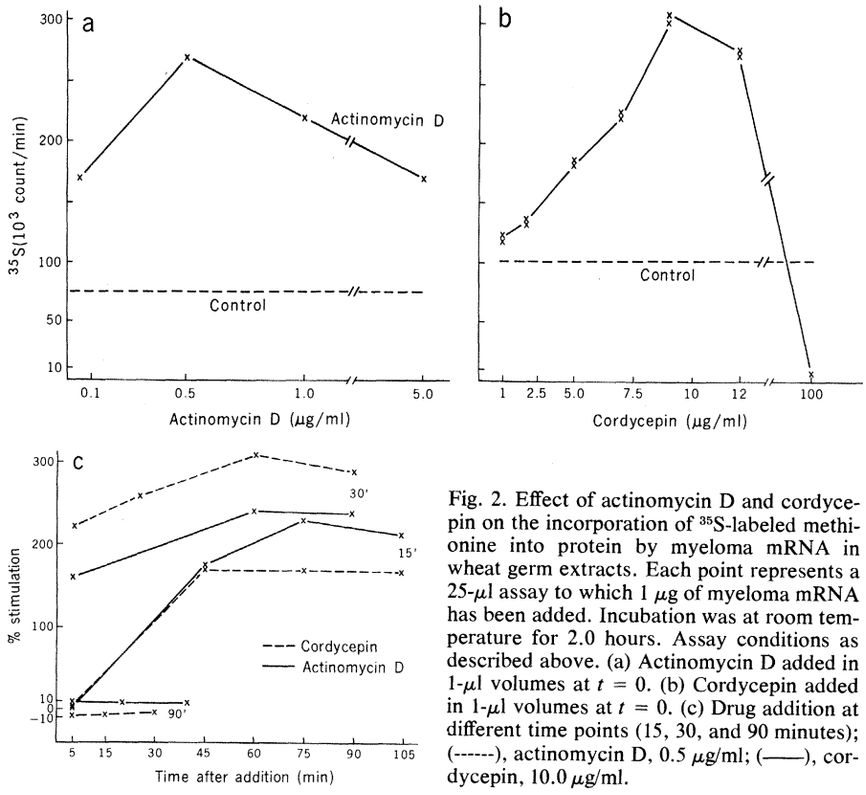

Fig. 2. Effect of actinomycin D and cordycepin on the incorporation of ^{35}S-labeled methionine into protein by myeloma mRNA in wheat germ extracts. Each point represents a 25-μl assay to which 1 μg of myeloma mRNA has been added. Incubation was at room temperature for 2.0 hours. Assay conditions as described above. (a) Actinomycin D added in 1-μl volumes at $t = 0$. (b) Cordycepin added in 1-μl volumes at $t = 0$. (c) Drug addition at different time points (15, 30, and 90 minutes); (------), actinomycin D, 0.5 μg/ml; (——), cordycepin, 10.0 μg/ml.

onine into protein, directed by myeloma mRNA in wheat germ extracts (Fig. 1). Actinomycin D and cordycepin also had a significant stimulatory effect on the translation of TMV RNA in the wheat germ system (data not shown). The maximum stimulation by actinomycin D and cordycepin was obtained at concentrations generally used to inhibit transcription of mRNA in intact cells (*7*). (It is not clear whether intracellular concentrations of the drugs reach these levels).

Concentrations of actinomycin D to 0.5 μg/ml or cordycepin in 10 μg/ml stimulated protein synthesis (Fig. 2, a and b), but increasing the drug concentration further produced less stimulation. Stimulation occurred at all concentrations of actinomycin D tested, but cordycepin inhibited translation at 100 μg/ml. The effect of the drugs was not additive. The maximum stimulation obtained in any combination of either drug was twofold. Therefore, the two drugs appear to be affecting the same process.

It is unlikely that the stimulatory effect of both drugs on translation is due to drug-induced changes in pH or ionic conditions. The pH and the K^+ and Mg^{2+} concentrations have been adjusted for each RNA to produce optimal incorporation. Since the optima have very sharp peaks, any shift in pH or ionic strength would decrease the efficiency of translation. Furthermore, neither drug at the concentrations tested changed the pH of the reaction mixture.

In order to investigate whether compounds of chemical structure similar to cordycepin could also stimulate the translation of myeloma or TMV mRNA's, the effect on translation of several nucleotide bases in the wheat germ system was measured. Adenosine, cytosine, guanosine, and uridine in concentrations of 10 μg/ml were tested separately to see whether they affected translation. The addition of a nucleotide base did not affect the incorporation of [^{35}S]methionine into protein when directed by myeloma RNA or TMV RNA. The same result was obtained in the presence of 10 μg/ml of the following deoxynucleotides: 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine, and 2′-deoxyuridine.

To determine whether the stimulatory effect of actinomycin D and cordycepin was unique to incorporation in the wheat germ system, endogenous activity or protein synthesis in the reticulocyte system was measured. The reticulocyte assays contained hemin ($4 \times 10^{-5}M$). The incorporation was linear up to 35 minutes, at which time the rate decreased; a plateau was reached by 50 minutes. Activity was measured in the presence and absence of actinomycin D and cordycepin. The drugs stimulated both the rate and extent of protein synthesis but not the time at which a plateau was reached. The stimulation by the drugs was approximately twofold.

Both drugs caused a marked stimulation in incorporation by myeloma mRNA when added at 15 or at 30 minutes after wheat germ assay incubation had begun (Fig. 2c). The response to the drug was much more rapid at 30 minutes than at 15 minutes, but, when added 90 minutes after translation had begun, neither drug affected incorporation even though the system was still actively synthesizing polypeptides. This suggests that the effect of the two drugs is not primarily that of reinitiation of mRNA molecules, since the system is still active at 90 minutes.

In order to determine whether these drugs stimulated the translation of specific messages or messengers of a specific size class, the proteins synthesized in translation assays were analyzed by polyacrylamide gel electrophoresis. Figure 3 shows densitometric tracings of an acrylamide gel autoradiograph of protein products from the translation of myeloma mRNA in wheat germ assays in the presence and absence of actinomycin D at 0.5 μg/ml. The synthesis of all polypeptide products was stimulated by both drugs. The tracings showed that the drugs did not change the relative proportions of proteins made by myeloma mRNA or TMV RNA, or endogenous reticulocyte mRNA (data not shown).

One possible mechanism of action of the two drugs is that they act at the level

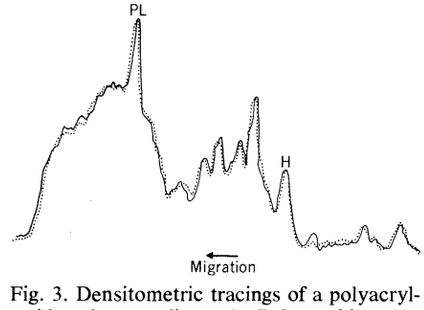

Fig. 3. Densitometric tracings of a polyacrylamide-gel autoradiograph. Polypeptides synthesized in the translation of 1 μg of myeloma mRNA in wheat germ extract were assayed in the presence and absence of drug. H and PL denote markers of heavy- and precursor light-chain immunoglobulin of molecular weight 55,000 and 30,000, respectively. The gel system is a modification of the method of Laemmli (*12*). It consists of a separating gel with an 8 to 20 percent gradient and a 4 percent stacking gel. Portions from translation assays were mixed with an equal volume of gel sample buffer (0.0625M tris, pH 8.8, 4 percent sodium dodecyl sulfate, 20 percent glycerol, and 10 percent β-mercaptoethanol) and boiled for 10 minutes prior to application to the gel; gels were subjected to electrophoresis at 70 volts for 18 hours. (——), One microgram of mRNA; (------), 1 μg of mRNA + 0.5 μg of actinomycin D per milliliter. Equal numbers of counts were applied to each slot.

of RNA secondary structure. It has been shown that changing the secondary structure of bacteriophage f2 RNA by treatment with formaldehyde increases its initiation efficiency. This was assayed by measuring the binding of formylmethionine (fmet)-tRNA to *Escherichia coli* ribosomes (*8*). In order to study the effect of changes in RNA secondary structure on the rate and extent of protein synthesis and the possible relation of these changes to drug treatment, both myeloma mRNA and TMV RNA were heated before translation. When the RNA was heated for 3 minutes at 60°C in 28 m*M* Hepes, *p*H 7.1, or distilled water and chilled immediately, the rate and extent of protein synthesis produced by heat-treated RNA's were increased up to twofold as compared to the controls which contained RNA that had not been heated. Neither drug stimulated the translation of heat-treated RNA further. Heating mRNA changes its secondary structure, possibly making it more available for translation. The rapid chilling of the samples prevents the reforming of those structures. The fact that actinomycin D and cordycepin do not further stimulate translation once the RNA has been heated suggests that both drugs may be altering the secondary structure of RNA.

These in vitro studies show that actinomycin D and cordycepin do affect the incorporation of [^{35}S]methionine into protein. Although we do not understand the basis for this phenomenon, it is not restricted to one mRNA nor to one in vitro protein synthesis system. Before it is possible to extrapolate these findings to in vivo translation, studies should be done on intact cells and on other in vitro systems. However, our experiments demonstrate a significant effect of actinomycin D and cordycepin on in vitro translation which should be taken into consideration in the use of these drugs.

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20 December 1976; revised 29 March 1977

Overlapping Platelets: A Diffusion Barrier in a Teleost Swimbladder

Abstract. *Overlapping platelets are layered within the connective tissue of the wall of a closed (physoclistous) swimbladder. The close, staggered arrangement of the platelets is viewed as a physical barrier that can interfere with the diffusion pathway of gas molecules. The result is a more efficient retention of gas pressures within the swimbladder.*

Fishes with swimbladders demonstrate remarkable variation in their abilities to actively secrete free gases into the organ. Physostomous fishes retain a functional connection—the pneumatic duct—between the swimbladder and the alimentary tract. Physoclistous fishes lose the connection during development. Gas secretion has been demonstrated in many physostomes; however, it is among the physoclists that gas-secreting abilities are most highly developed. The gas-filled luminal space, important for hydrostatic equilibrium, is maintained against the pressure of water at the depth in which a fish may live. This pressure increases approximately 1 atm per 10 m of depth. Fishes that secrete oxygen against great pressures, particularly those that inhabit a depth of 1000 m or more, are known to have as much as 85 to 95 percent oxygen within the swimbladder lumen (*1, 2*). Thus, oxygen pressure approaches 100 atm and oxygen transport is against a considerable gradient (*1, 3–5*). Fishes die quickly when exposed to water in which dissolved oxygen is equivalent to elevated oxygen pressures found within their own swimbladders (*6*). Oxygen pressures in excess of 5 atm are generally toxic to living tissues (*7*). Many fishes—particularly physoclists—are therefore faced to a varying degree with two physical necessities: (i) the secretion of free gases against pressure and (ii) the retention of those gases within the swimbladder lumen in order to minimize the load on the secreting mechanism and, in many cases, to prevent diffusion pressures that may reach toxic levels. The former problem has received considerable attention (*3, 8, 9*), the latter, relatively little. It has been shown, however, that the swimbladder wall can be 100 times less permeable to gaseous diffusion than normal connective tissues (*10*). Crystalline material, composed principally of guanine, in an outer layer of the swimbladder wall has been described as a barrier against diffusion in a few species (*5, 10, 11*). In other species, dense collagen is believed to be a barrier (*12*). We now describe another structure that may provide a physical barrier to gaseous diffusion in the swimbladder walls of the mummichog (*Fundulus heteroclitus*), gulf killifish (*Fundulus grandis*), sheepshead minnow (*Cyprinodon variegatus*), and sailfin molly (*Poecilia latipinna*).

The luminal surface of a typical swimbladder wall is bounded by a layer of epithelial cells. Anteriorly, the epithelium is thickened, highly vascularized, and secretory in nature. The rete mirabile, a countercurrent exchange multiplier of blood gases (*13*), is most anterior. These two tissues are implicated in the functional release of gases into the lumen (*8, 14*).

Within the submucosal region of the swimbladder wall (*15*) in those species we have investigated, can be seen round, flattened platelets (Figs. 1 and 2) dispersed throughout the loose fibroelastic connective tissue. The platelets vary in diameter from approximately 10 to 80 μm, although most are in the range between 40 to 60 μm. Many, but not all, exhibit a single nucleus that is most often centrally located. A minute central hillock is seen on the surface of many of the platelets. Since adjacent platelets consistently overlap one another by one-half of their diameters, the hillock may have the function of preventing complete superimposition. Such an orderliness would increase the efficiency of any given number of platelets as diffusion barriers.

The entire swimbladder wall thickens and the relative abundance of platelets is greatest about the gas-secreting mechanisms. Posteriorly, the wall is relatively thin, vascularization is much reduced, and the platelets are less concentrated. Light-microscope observations of whole tissue can be made most easily in this region, and the platelets can be clearly seen in face view (Figs. 1 and 2). Phase-