centrifuged (45,000 rev/min, 1 hour, SW-50 rotor, Spinco L2 ultracentrifuge). The single mixed band of cores and reaction cores that formed in the lower portion of the tube was collected by piercing the side of the centrifuge tube with a hypodermic syringe of small bore; the sample was then diluted fourfold with 1 percent formaldehyde. Cytochrome c was added to give a final concentration of 100 μg/ml, and the mixture was immediately spread (*8*) over a hypophase of 0.2*M* ammonium acetate, *p*H 6.8, containing 0.5 percent formaldehyde. The monolayer was transferred to grids that had been coated with collodion and stabilized with a carbon film. The grids were then rotary shadowed with platinum-palladium from an angle of approximately 5 degrees.

When purified formaldehyde-fixed reaction cores were spread and examined by election microscopy, RNA strands were clearly visible (Fig. 1, a–f). The following evidence strongly supports the contention that the structures shown in Fig. 1, a–f, are reaction cores.

1) Lysed particles and free double-stranded RNA derived from them were removed prior to spreading and electron microscopy by sedimentation into the density gradient.

2) RNA strands were not observed associated with cores if transcriptase reactions were carried out in the absence of ATP [RNA synthesis by cores required the presence of all four ribonucleoside triposphates (Fig. 1g)].

3) RNA strands were not observed associated with reaction cores which had been digested with pancreatic ribonuclease A after incubation and prior to fixation and spreading (10 μg/ml for 10 minutes at 39°C, 0.15*M* salt, $10^{-3}M$ $MgCl_2$).

4) When single-stranded RNA and purified cores were mixed and then spread, structures such as those shown in Fig. 1, a–f, were not observed, implying that released single-stranded RNA does not reassociate with cores to form artifacts resembling reaction cores.

We have estimated the proportion of viral cores which participate in the polymerase reaction by counting particles which show evidence of attached messenger RNA. In a random count of 8100 particles were observed 695 (8.5 percent) reaction cores. The small proportion of active cores observed here is in agreement with other data (*9*) indicating that reovirus cores synthesize approximately eightfold less RNA than the amount expected from the known rate of chain elongation. However, some aspects of our preparative procedure might influence the accuracy of our estimate of the proportion of active cores. For example, reaction cores show a marked tendency to aggregate (Fig. 1a), and this might prevent observation of much of the RNA present.

Examination of reaction cores that showed visible strands gave an average of 2.8 strands (free-ends) per particle. Occasionally two or more strands were observed coiled around each other (Fig. 1f). Frequently this would result in their being scored as one strand. The maximum number of strands observed associated with a core particle was ten, a value in agreement with the number of messenger RNA molecules produced by the transcriptase (*1, 2*).

Further studies will be required to provide a more detailed structural analysis of reovirus reaction cores. However, examination of the electron micrographs presented in Fig. 1 permit two preliminary conclusions to be reached concerning the synthesis of messenger RNA by the reovirus RNA transcriptase. (i) At least some of the cores appear capable of synthesizing ten messenger RNA molecules. It is probable that these strands represent the ten individual messengers synthesized in vitro (*1, 2*). (ii) Nascent messenger RNA molecules appear to be attached to different sites on the surface of the core, suggesting that more than one enzymic site exists per core.

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Acridine Orange Potentiation of Actinomycin D Uptake and Activity

Abstract. *Acridine orange enhances the uptake of [^3H]actinomycin D in disrupted and intact human lymphocytes, as measured by liquid scintillation and autoradiography. Proflavine, quinacrine, chloroquine, and ethidium bromide are not effective. In mice, acridine orange increases the capacity of actinomycin to reduce spleen weight. Type II acridine binding to DNA may be a prerequisite for actinomycin enhancement.*

Actinomycin D is a potent inhibitor of DNA-dependent RNA synthesis and an effective agent in the treatment of certain malignant tumors. Although it has a marked effect on lymphoid tissues, autoradiographic studies of [^{3}H]-actinomycin D uptake have shown that only 5 to 28 percent of human lymphocytes in culture take up actinomycin (*1*). These studies also demonstrated that, 24 hours after stimulation of the lymphocytes with phytohemagglutinin, there occurred a large increase in the number of cells binding actinomycin. It is likely that an agent that could enhance the uptake of actinomycin would potentiate its activity and possibly its antitumor effect as well. This report shows that acridine orange is capable of enhancing the uptake of actinomycin in vitro and its activity in vivo. Unlike phytohemagglutinin, its effect appears rapidly and is not dependent on intact cellular processes.

Acridine orange was obtained from Calbiochem and the National Aniline Corporation; proflavine, chloroquine, quinacrine, and ethidium bromide from the Sigma Chemical Company.

Blood from normal donors was collected in phenol-free heparin. The red cells were sedimented with 5 percent dextran, and the leukocyte-rich plasma was passed through a column of washed Fenwal Leukopak nylon. The yield of approximately 95 percent lym-

phocytes (with at least 90 percent viability as determined by the acridine orange test) (*2*) was suspended in tris-buffered minimal essential medium (*p*H 7.3) 15 percent fetal calf serum, 1 percent glutamine, and penicillin-streptomycin (all from Microbiological Associates, Bethesda, Maryland). Lymphocytes to be disrupted by freeze-thawing and sonication were suspended in tris-buffered isotonic saline, *p*H 7.3. The cell concentration was adjusted to 2×10^6 cells per milliliter by means of a Coulter counter, model B.

Samples (0.5 ml) of intact or disrupted cells were incubated at 37°C for 1 hour with 0.2 μc of [^{3}H]actinomycin D (Schwarz/Mann Company, Orangeburg, New York; specific activity, 8.4 c/mmole). A 1-hour incubation time was chosen because, in this system, actinomycin uptake increases rapidly up to 1 hour and levels off thereafter. Acridine orange or other acridine derivative was added just prior to actinomycin. In the experiments comparing the effects of different acridine derivatives, they were all used at a final concentration of $4 \times 10^{-4} M$. All determinations were done in duplicate. After incubation in the dark, the cells were trapped on glass fiber Whatman GF/C filters after the method of Sorensen *et al.* (*3*) and washed with 160 ml of isotonic saline and 40 ml of methanol. The filters were prepared in hyamine for liquid scintillation counting in toluene containing 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl]-benzene on a Packard Tri-Carb counter. Quench correction was performed by the external standard method, and the counts were expressed as disintegrations per minute (dpm) per 10^6 cells.

Autoradiographs were prepared by coating washed smears of the lymphocyte suspensions with Kodak NTB2 emulsion. These were exposed for 1 week and developed by standard methods. One thousand cells were counted for each experiment.

Male B6D2F1/J mice, 4 to 6 weeks old, were obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals received a single intraperitoneal injection of saline, acridine orange, and actinomycin or acridine orange followed by actinomycin. The mice were killed 72 hours later, and spleen weights were determined.

Table 1 shows the effect of two concentrations of acridine orange on actinomycin uptake by intact or freeze-thawed human lymphocytes disrupted by ultrasound. At a concentration of 0.100 mg/ml, acridine orange produced approximately threefold increases in actinomycin uptake. It is noteworthy that whereas cell disruption alone resulted in a marked increase in actinomycin uptake, the proportional increases produced by acridine orange were similar in both the intact and disrupted cells. These increments above the control were highly significant ($P < .001$). Comparison of the effect of four other acridine derivatives (proflavine, quinacrine, chloroquine, and ethidium bromide) at equimolar concentrations showed that none of these compounds produced a significant increase in actinomycin uptake. Table 2 gives the results of two autoradiographic determinations of actinomycin uptake by human lymphocytes. In the control cells, only 15 percent have at least three grains per cell, whereas at concentrations of acridine orange of 0.100 mg/ml over 50 percent showed this degree of labeling. In addition, cells treated with acridine orange showed an eightfold increase in heavily labeled cells with five or more grains per cell.

Table 3 shows the effect in vivo of actinomycin and acridine orange on the size of mouse spleen. A single injection of actinomycin at 0.100 mg per kilogram of body weight failed to produce a statistically significant reduction in spleen size. However, when given with acridine orange at 50 mg/kg, actinomycin produced a mean reduction of 25 percent in spleen weight. This difference is significant at less than the 0.1 percent level. Acridine orange alone produced no change in spleen weight.

These studies show that lymphocytes previously treated with acridine orange take up significantly more actinomycin. The effect of acridine orange is dose-related. Of the five acridine derivatives tested, only acridine orange potentiated the uptake of actinomycin. This effect was also evident in disrupted cells, indicating that a change in cell permeability is an unlikely explanation for the enhancement of actinomycin uptake. Autoradiography showed that only about 15 percent of normal resting lymphocytes bound significant amounts of actinomycin (three or more grains per cell). The addition of acridine orange recruited an increased number of labeled cells. At acridine concentrations of 0.100 mg/ml, over 50 percent of the cells were significantly labeled by the same criteria. Correspondingly, in vivo, a dose of actinomycin which by itself caused no change in the size of the normal mouse spleen produced a significant shrinkage when given with acridine orange.

Actinomycin has been shown to have a high specificity for double-stranded DNA. However, the exact sites of bonding remain controversial (*4*). Both intercalation of the drug between base pairs and attachment to the amino groups of guanine in the minor groove of the DNA helix have been proposed (*5*). These views are not mutually exclusive since at least two different types of binding have been demon-

Table 1. The effect of acridine orange on the uptake of [^{3}H]actinomycin D by intact and disrupted human lymphocytes. There were 24 determinations with intact lymphocytes and 12 with the disrupted ones. For intact lymphocytes, 100 percent equals 5280 dpm per 10^6 cells; for disrupted lymphocytes, 100 percent equals 42,704 dpm per 10^6 cells. Values are given ± the standard deviation.

Control (%)	Acridine orange	
	0.040 mg/ml	0.100 mg/ml
Intact lymphocytes		
100 ± 26.9	167 ± 27.2	268 ± 93
Disrupted lymphocytes		
100 ± 34.1	123 ± 11.1	317 ± 83.8

Table 2. Autoradiography of [^{3}H]actinomycin D uptake by normal human lymphocytes and those treated with acridine orange from two donors (A and B). Concentration of acridine orange, 0.100 mg/ml. Values are expressed as percentage per 500 cells.

Donors	Grains per cell		
	0 to 2	3 to 4	5 or more
	Control		
A	86.2%	11.4%	2.4%
B	84.6%	13.4%	2.0%
	Acridine orange		
A	49.3%	34.4%	16.3%
B	46.9%	36.4%	16.7%

Table 3. Effect of actinomycin D (0.100 mg/kg) and acridine orange (50 mg/kg) on normal mouse spleen weight, expressed in milligrams ± standard deviation. Numbers of mice are given in parentheses.

Treatment	Spleen weight (mg)
Saline (control) (15)	41.1 ± 10
Acridine orange (15)	39.9 ± 11
Actinomycin D (21)	37.3 ± 6.8
Actinomycin D + acridine orange (21)	30.0 ± 5.0

strated and the association constants measured (*6*). Both actinomycin and acridine orange binding to DNA are inhibited by divalent cations (*7*). Unlike the bonds between acridine and DNA, those between actinomycin and DNA are thought to be of the hydrogen bond type (*8*).

The details of acridine orange–DNA binding have been described by Bradley and Wolf (*9*). They have characterized two types of binding of acridines to DNA. Type I, which is common to all acridines, is the intercalation model based on the x-ray crystallography studies of Lerman (*10*). This bond has strong electrostatic forces of the order of 6 to 10 kcal/mole and is relatively resistant to divalent cations. The plane of the bound acridine molecules is approximately perpendicular to the DNA axis. Type I binding is favored by double-stranded DNA and results in untwisting and lengthening of the helix. Type II binding is a predominant characteristic of only a few dyes of which acridine orange is usually cited as the chief example. The binding site is external to the helix. Acridine molecules are thought to attach to the helix with the positively charged ring nitrogen close to the negatively charged deoxyribose phosphate groups. The bonding forces are weaker, favored by a disordered or single-stranded DNA and readily affected by divalent cations. Type II binding results in stacking of aggregates of acridine molecules whose number is limited by the number of charged residues. Since all the acridine derivatives tested here exhibit type I binding (intercalation) and only acridine orange is effective, it is clear that intercalation per se is not sufficient to potentiate actinomycin uptake. Type II binding, however, which is a particular feature of acridine orange, may be a necessary prerequisite for this effect. Both type I and type II binding of acridine have in common the fact that the positively charged ring nitrogen is in apposition to the negatively charged oxygen atoms of the backbone phosphate groups. The negative charges of the phosphate groups tend to repel each other in native DNA and unless opposed by counterions tend to unwind the DNA molecule (*11*). The interaction of aminoacridine and DNA appears to favor areas of adjoining purine base pairs rather than pyrimidine pairs (*12*), so that the apposition to negative phosphate groups is not random. The purine base guanine was selected by Reich (*4*) as the probable site of actinomycin attachment to DNA. It is possible that the steric alterations produced by acridine orange in this location could shift additional guanine residues to the precise configuration necessary for actinomycin binding.

Although actinomycin D is presently used in the treatment of Wilms' tumor, Ewing's tumor, rhabdomyosarcoma, and a number of anaplastic sarcomas (*13*), it is of little therapeutic value in the treatment of leukemia, lymphoma, and other commonly occurring neoplasms. The enhancement of actinomycin uptake by acridine orange may broaden the spectrum and efficacy of this antineoplastic agent.

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Adult Hemoglobin Synthesis by Reticulocytes from the Human Fetus at Midtrimester

Abstract. *The synthesis of adult-type hemoglobin was measured in small samples of peripheral blood cells from 9- to 18-week human fetuses. Hemoglobin indistinguishable from hemoglobin A was identified by ion-exchange chromatography, electrophoresis at pH 8.6, tryptic peptide analysis, and the insensitivity of its synthesis to the action of O-methylthreonine. Synthesis of hemoglobin A accounted for 8 to 14 percent of total hemoglobin synthesis and was demonstrated in as little as 10 microliters of fetal blood. These studies provide sensitive methods for the detection of β chain types in hemoglobin synthesized by the human fetus at midtrimester. If methods to obtain small quantities of fetal blood at midtrimester become available, these techniques should be applicable to the antenatal detection of sickle cell anemia and related hemoglobinopathies.*

Hemoglobin F ($\alpha_2 \gamma_2$) comprises 70 to 90 percent of human hemoglobin at birth (*1*), while hemoglobin A ($\alpha_2 \beta_2$) is the predominant hemoglobin in the adult. Differences in the amino acid sequences of the γ chain of hemoglobin F and the β chain of hemoglobin A have been elucidated (*2*). Since mutations of the β chain are responsible for most hemoglobinopathies observed in man, knowledge of β chain production in the early fetus is critical for the antenatal detection of these conditions. Little information is available regarding the synthesis of adult hemoglobin during this period of human development.

In sheep (*3*) and mice (*4*) adult-type hemoglobin appears early in fetal development. In the human, data suggesting the presence of hemoglobin A in the maturing fetus (25 to 70 mm crown-to-rump) has been reported (*5*). Walker and Turnbull used alkali denaturation to determine the proportion of hemoglobin other than hemoglobin F in the human fetus aged 11 to 43 weeks (*6*). No alkali-denaturable hemoglobin was detected in blood samples from four fetuses aged 11 to 12 weeks. At week 13, 1 to 2 percent of the total hemoglobin was alkali-sensitive, and presumably this hemoglobin was other than hemoglobin F. The proportion of alkali-sensitive hemoglobin increased to 10 percent by weeks