the toxicity of the other parts (*15*). Foot and muscle, which have a locomotor function, change the toxin profile conspicuously. Such locomotor tissues have large amounts of the enzymes that participate in the redox reactions.

The toxic shellfish samples analyzed for the toxin contents have shown various ratios of saxitoxin to other toxins (*5, 6*). In some cases, such as a *Mytilus* sp. from Haines, Alaska, saxitoxin was almost absent, whereas other specimens from nearby waters contained significant amounts of saxitoxin (*16, 17*). Our findings, although limited to experiments in vitro, can at least partly explain these discrepancies.

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15 September 1980; revised 15 December 1980

Tritiated Thymidine Incorporation Does Not Measure DNA Synthesis in Ribavirin-Treated Human Cells

Abstract. *When the incorporation of tritiated thymidine into acid insoluble material was measured, ribavirin appeared to be a potent inhibitor of DNA synthesis in KB cells and human lymphocytes. Inhibition was nearly 100-fold less, however, when DNA synthesis was measured by incorporation of phosphorus-32–labeled phosphate or by DNA fluorescence. The potent inhibition detected by incorporation of tritiated thymidine into DNA actually was the result of a potent effect on the labeling of deoxythymidine triphosphate, not on the synthesis of DNA.*

The mode of action of the antiviral drug ribavirin (*1, 2*) involves inhibition of nucleic acid biosynthesis (*3–7*). The effect of ribavirin on DNA synthesis in uninfected mammalian cells appears to be especially potent when measured by incorporation of [^{3}H]thymidine into acid precipitable material. As little as 2 μM ribavirin produces 50 percent inhibition in primary (*1, 3*) and established (*5, 6*) cell lines from various species. It is difficult to explain such potent inhibition of DNA synthesis, however, on the basis of inhibition of the enzymes (*8*) known to be affected by ribavirin. Furthermore, potent inhibition of DNA synthesis is not consistent with the low cytotoxicity of the drug in cell culture (*1, 2, 9*) and with the low toxicity observed in animals and humans (*1, 2*). To resolve this inconsistency, we have reexamined the effects of ribavirin on both DNA synthesis and [^{3}H]thymidine metabolism in KB cells.

We incubated monolayer cultures of KB cells with selected concentrations of ribavirin or vidarabine (ara-A)—a known inhibitor of DNA synthesis (*5*). Both [^{3}H]thymidine and [^{32}P]phosphate were used as precursors to measure DNA synthesis. Figure 1 shows that ara-A had nearly the same effect on the incorporation of [^{3}H]thymidine and [^{32}P]phosphate into DNA. In contrast, the effect of ribavirin was much more potent on [^{3}H]thymidine incorporation than it was on [^{32}P]phosphate incorporation, suggesting the two precursors measured different events. We also examined the effect of ribavirin on incorporation of [^{3}H]thymidine and [^{32}P]phosphate into concanavalin A–stimulated lymphocytes. The experimental protocol was similar to that described in the legend to Fig. 1; other techniques have been detailed by Lopatin *et al.* (*10*). Results obtained with lymphocytes were virtually identical to those obtained with KB cells (data not shown). The concentrations of ribavirin that inhibited [^{3}H]thymidine and [^{32}P]phosphate incorporation by 50 percent (I_{50}) were 5 μM and 225 μM, respectively. These concentrations correlate well with the I_{50} concentrations of 3 μM and 230 μM interpolated from the data for KB cells in Fig. 1.

To explore why ribavirin produced such different effects on [^{3}H]thymidine and [^{32}P]phosphate incorporation, we examined the metabolism of [^{3}H]thymidine in ribavirin-treated KB cells. Several concentrations of ribavirin were incubated with duplicate monolayer cultures of KB cells. To each flask we added [^{3}H]thymidine at selected times; then we extracted the radioactive intracellular nucleotides and characterized them chromatographically as described in the legend to Fig. 2. Ribavirin (131 μM) caused a protracted decline in the labeling of deoxythymidine triphosphate

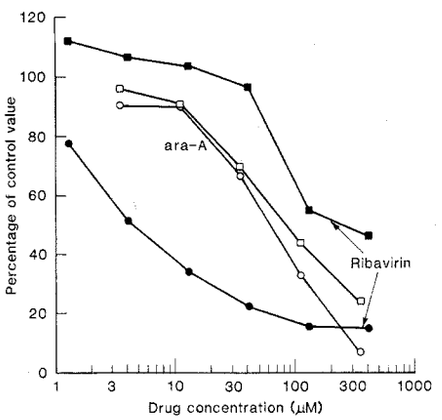

Fig. 1. Effect of ribavirin and vidarabine (*ara-A*) on the incorporation of labeled precursors into cellular DNA. Monolayer cultures of KB cells in 25 cm^2 plastic tissue culture flasks were incubated at 37°C for 14 hours in the presence of selected concentrations of ribavirin (closed symbols) or vidarabine (open symbols) plus [Me-^{3}H]thymidine (2 μCi/ml; 5 μM) (○, ●) and carrier-free [^{32}P]orthophosphate (5 μCi/ml) (□, ■). After the incubation period, the cells were harvested, and cell pellets were dissolved in 1 ml of 0.3*N* KOH and incubated at room temperature for 72 hours to hydrolyze RNA. Portions (100 μl) of the KOH solutions were spotted in triplicate onto filter paper disks which were processed as described previously (*14*) for determination of label incorporated into DNA. For other experimental details, see (*5*). In the absence of drugs, the radioactivity incorporated per 100 μl of cell solution was as follows: 67,600 count/min for [^{3}H]thymidine and 78,900 count/min for [^{32}P]phosphate. All data points represent arithmetic means of results from duplicate cultures.

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(dTTP) and an increase in the amount of intracellular [^{3}H]thymidine; only minor effects were noted on the concentrations of ^{3}H-labeled deoxythymidine monophosphate (dTMP) and deoxythymidine diphosphate (dTDP) (Fig. 2). Lower drug concentrations were less effective but produced similar time-dependent declines in the labeling of dTTP. The amount of [^{3}H]thymidine incorporated into an acid insoluble form also was quantitated in this experiment and showed the same potent inhibition by ribavirin as that depicted in Fig. 1.

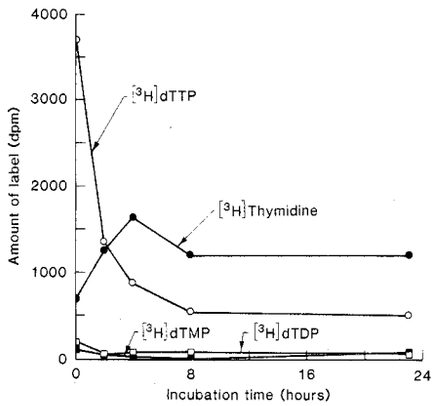

Fig. 2. Effect of ribavirin on labeling of thymidine nucleotides. Monolayer cultures of KB cells in 25-cm^2 plastic flasks were prepared as described (*5*) and incubated with 131 μM ribavirin at 37°C. At the times indicated, [^{3}H]thymidine was added to duplicate flasks at a final concentration of 2 μCi/ml and incubation was continued for an additional 15 minutes. Medium then was aspirated, the cell sheets rinsed with 1 ml of cold Hepes buffered saline (*15*) and the cells removed with 0.5 ml of cold 25 mM EDTA, pH 8.0. The resulting cell suspensions were quantitatively transferred to conical tubes and the flasks rinsed two times with 0.5 ml of 95 percent ethanol. The rinses were added to the cell suspensions. The suspensions were mixed vigorously, sonicated, and triplicate 100-μl portions were spotted on filter paper disks for the determination of incorporated label (*14*). After standing 12 hours or more at −20°C, precipitated materials were removed by centrifugation and 10-μl portions were spotted along with unlabeled standards onto polyethyleneimine (PEI) cellulose thin-layer sheets. The sheets were developed using the first dimension of a chromatographic system described by us (*16*). The sheets were sectioned, eluted, and counted as described previously (*17*) with the exception that 1 to 2 ml of 1M $MgCl_2$ was used to facilitate complete elution of labeled nucleotides from the PEI-cellulose sections.

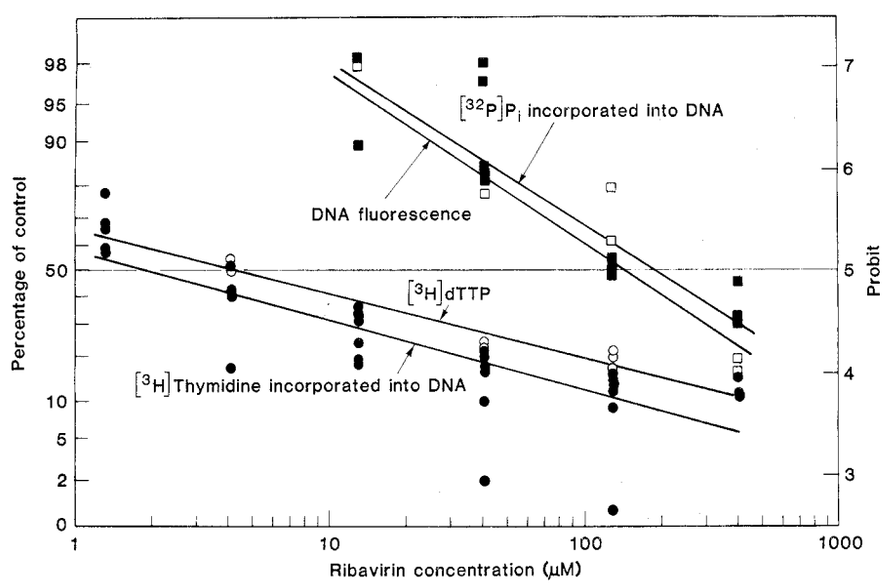

Fig. 3. Dose-response relationships between ribavirin concentration and parameters used to measure DNA synthesis. DNA fluorescence data were derived by incubating duplicate cultures of KB cells for 12 hours with selected concentrations of ribavirin. The protocol described in the legend to Fig. 1 was used, except that [^{3}H]thymidine was present during only the last one-half hour. In addition, duplicate flasks without drug were held at 2°C during the 12-hour incubation period to provide data on the amount of DNA present prior to the incubation period. After incubation, cells from all cultures were harvested and portions of suspended cells were assayed for cell number and incorporated [^{3}H]thymidine by techniques described previously (*5*, *17*). The remainder of the cells were pelleted by centrifugation, lysed by resuspension in 0.1 percent sodium citrate containing propidium diiodide (50 μg/ml), and examined in a Coulter model TPS-1 flow cytometer according to the procedure of Krishan (*18*). Relative amounts of DNA synthesized in each culture during the incubation period were determined as described (*11*). The dose-response relationship was derived by linearly regressing probit values of percent inhibition of DNA synthesis against log drug concentration (*12*). Derivation of the other data plotted in the figure are described in the text or other figure legends. Inhibition data were obtained in the following number of replicate experiments: determination of DNA fluorescence (□), two experiments; determination of [^{32}P]phosphate incorporation (■), three experiments; determination of [^{3}H]dTTP levels (○), three experiments; and determination of [^{3}H]thymidine incorporation (●), six experiments.

Because these data indicated that most of the effect of ribavirin on [^{3}H]thymidine incorporation was a result of its effect on the formation of [^{3}H]dTTP, we used a more direct means to quantitate the effect of the drug on DNA synthesis. We measured in a flow cytometer the ultraviolet light-induced fluorescence from the uptake of propidium diiodide by DNA, using nuclei from cells treated with ribavirin as described in the legend to Fig. 3. Histograms of DNA fluorescence plotted against cell number revealed that only the highest concentration of ribavirin tested (410 μM) caused a detectable portion of cells in G_1 phase of growth to shift toward S phase (data not shown). Relative amounts of DNA synthesized during the incubation period were determined from the areas under the histograms (*11*). The amounts were converted to percentage inhibition, linearly regressed against log drug concentrations (*12*), and are presented as a dose-response line in Fig. 3. The I_{50} concentration interpolated from the dose-response line was 146 μM [95 percent confidence interval, 77 to 402 μM (*12*)].

The effect of ribavirin on fluorometrically measured DNA synthesis was compared to its effect on DNA synthesis measured by incorporation of [^{3}H]thymidine and [^{32}P]phosphate into acid precipitable material. Data presented in Fig. 1 were combined with additional results from similar experiments. In the additional experiments, both monolayer cultures and stirred suspensions of KB cells were incubated with ribavirin for 10 to 16 hours. Data from all such experiments were regressed against log drug concentrations (*12*) and are summarized as dose-response lines in Fig. 3. The I_{50} concentrations interpolated from the dose-response lines were 1.9 μM (1.2 to 3.0 μM) for inhibition of [^{3}H]thymidine incorporation and 192 μM (146 to 252 μM) for inhibition of [^{32}P]phosphate incorporation. The I_{50} concentration range for inhibition of DNA fluorescence (77 to 402 μM) overlapped the 146 to 252 μM I_{50} concentration range for inhibition of [^{32}P]phosphate incorporation, but was markedly different from the 1.2 to 3.0 μM I_{50} concentration range for the inhibition of [^{3}H]thymidine incorporation. In addition, when the dose-response lines themselves were compared (*13*), the dose-response lines for inhibition of DNA fluorescence was the same as the line for inhibition of [^{32}P]phosphate

incorporation ($P = .85$), but was very different from the line for inhibition of [^{3}H]thymidine incorporation ($P < .0001$). From these data we conclude that the incorporation of [^{32}P]phosphate was a good measure of DNA synthesis in ribavirin-treated cells whereas the incorporation of [^{3}H]thymidine was not.

We also compared the foregoing data to the inhibitory effect of ribavirin on [^{3}H]dTTP formation. Data derived from Fig. 2 and from similar experiments at lower drug concentrations were regressed against log drug concentrations. The I_{50} concentration for inhibition of [^{3}H]dTTP formation interpolated from the dose-response line (Fig. 3) was 4.3 μM (2.7 to 6.8 μM). This dose-response line was similar to the line for inhibition of [^{3}H]thymidine incorporation into DNA ($P = .18$), but was significantly different from the lines for inhibition of [^{32}P]phosphate incorporation into DNA ($P < .0001$) and DNA fluorescence ($P < .0001$). On the basis of the similarity between effects of ribavirin on the incorporation of [^{3}H]thymidine into dTTP and DNA, we conclude that ribavirin has a potent effect on the labeling of dTTP and that this effect is primarily responsible for the inhibition of [^{3}H]thymidine incorporation into DNA. The marked inhibition by ribavirin of [^{3}H]thymidine incorporation in other primary (*1, 3, 4*) and established (*1, 5–7*) cell lines suggests the phenomenon extends to many types of mammalian cells.

Our data indicate that when DNA synthesis is measured by methods other than incorporation of [^{3}H]thymidine, ribavirin is a weak inhibitor in KB cells and human lymphocytes. The use of [^{3}H]thymidine to measure DNA synthesis is inaccurate because of the effect of ribavirin on [^{3}H]dTTP formation. These observations lead to the general premise that the sole use of [^{3}H]thymidine to measure DNA synthesis in drug-treated mammalian cells may cause serious misinterpretations of data.

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12. Dose-response relationships were constructed by linearly regressing probit values of parameters related to DNA synthesis against log drug concentrations. The I_{50} concentrations and corresponding 95 percent confidence intervals were calculated from the regression lines by using methods described by A. Goldstein [*Biostatistics: An Introductory Text* (Macmillan, New York, 1964), pp. 156–161].
13. Identity of any two dose-response curves was tested by calculating P values for the equality of the regressions and slopes as described by D. J. Fox and K. E. Guire [*MIDAS: Michigan Interactive Data Analysis System* (Univ. of Michigan Press, Ann Arbor, 1973), pp. 59–60]. The slopes of the immediately adjacent dose-response curves compared in this study were similar ($P > .80$), consequently P values given in the text refer only to the comparison of regressions.
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6 August 1980; revised 24 November 1980

Ctenidial Autotomy in *Corbicula fluminea* in Response to Massive Granulomas

Abstract. *Large granulomas (greater than 2 millimeters in diameter), stimulated by the presence of and formed around necrotic larval tissue in the inner demibranchs of* Corbicula fluminea, *are eliminated by autotomy. Granulocytes invade and destroy ctenidial epithelium adjacent to the granuloma, causing it and the granuloma to slough away into the mantle cavity, where they are removed as pseudofeces.*

Foreign material in the bodies of mollusks has been found to elicit one of three kinds of cellular defense mechanisms: phagocytosis, encapsulation, or nacrezation (*1*). We report here a fourth defense mechanism, autotomy, which is induced by the presence of massive hyperplasic granulomas encapsulating necrotic larval tissue in ctenidial (gill) marsupia of the bivalve *Corbicula fluminea* (Müller 1774). Morton (*2*) described the development of granulomas in the ctenidial marsupium of *C. fluminea* from Plover Cove, Hong Kong. Like many freshwater bivalves, *C. fluminea* retains the larvae within the ctenidia (inner demibranchs) before releasing them into the environment. Occasionally some larvae die while in the marsupium. If dead larvae are not moved from the ctenidial marsupia to the exterior via the suprabranchial chamber and excurrent siphon, they are encapsulated by epithelioid cells and cyst-associated granulocytes. Morton (*2*) described in detail the process of granuloma formation and concluded that amoebocytes "probably reabsorb the larval cellular debris." We have observed *C. fluminea* from several localities in California and Texas which bear similar larvae-induced ctenidial granulomas. At least some are removed, not by the activities of phagocytes, but by autotomy of the affected tissue.

We found that the incidence of ctenidial granulomas is closely correlated with clam size (Fig. 1a) and reproductive periodicity (Fig. 1, b and c) (*3*). Clams with shells longer than 30 mm frequently contain cysts up to 5 mm in diameter after the spring and fall peaks of larvae production. About 1 month after the appearance of the large granulomas, approximately 40 to 50 percent of the affected clams have torn, ragged inner demibranchs and few, if any, large granulomas (Fig. 1c). We observed material as large as 3 by 4 mm (subsequently identified as fragments of ctenidial tissue surrounding granulomas) expelled as pseudofeces from the mantle cavity through the incurrent siphon. Ctenidia examined at periodic intervals for 6 months after the loss of granulomas show progressive tissue repair, with eventual restoration of normal ctenidial size (Fig. 1c).

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