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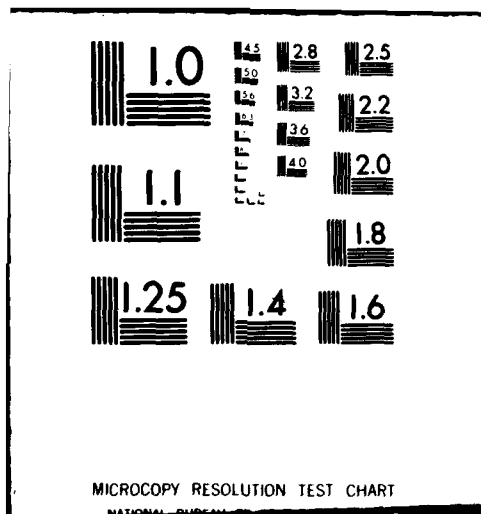
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6 MECHANISM OF ACTION OF RIBAVIRIN: AN
ANTIVIRAL DRUG OF MILITARY IMPORTANCE (U) 12 12

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a nucleoside analogue having broad spectrum antiviral activity against both DNA and RNA viruses (18). A variety of specific effects on host cell metabolism have been attributed to ribavirin or its metabolites. For example, ribavirin is reported to be a strong inhibitor of thymidine phosphorylation (6) and its 5'-monophosphate derivative (RMP) is a potent competitive inhibitor of inosine-5'-phosphate dehydrogenase (19). Ribavirin is also reported to decrease DNA, RNA and protein synthesis and reduce the size of the cellular guanosine-5'-triphosphate pool (5,12).

Numerous other reports, however, contradict many of the alleged cellular effects of ribavirin (2,14,15,17). As a result, the pharmacological mechanism of action of ribavirin remains obscure. It is not yet clear whether this compound is specifically antiviral or whether it inhibits virus replication as a result of its effects on host cellular metabolism.

The study reported here attempts to clarify the mode and specificity of action of ribavirin. We have examined its effects on cellular metabolism and on the replication of Venezuelan equine encephalomyelitis virus (VEE) grown in BHK-21 cells.

MATERIALS AND METHODS

Virus Stock and Plaque Assay: The live attenuated VEE vaccine, strain TC-83, was obtained in the lyophilized state from Merrill-National Laboratories (lot 4, run 2). It was reconstituted with 1.2 ml

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of sterile water, and passaged once in primary duck embryo cell cultures (DEC), concentrated, and purified by rate zonal centrifugation in 10 to 30% sucrose gradients, as described previously (1). Infectious virus was titrated by counting plaque forming units (pfu) in DEC monolayers grown in 10-cm² plastic plates, and maintained in a humidified atmosphere with 5% CO₂ under medium containing 1% agarose, as described previously (9).

Cell Line and Infection: Baby hamster kidney cells (BHK-21, clone 13) were obtained from the American Type Culture Collection, and used at passage levels varying between 55 and 70. To infect cells, concentrated TC-83 virus was diluted in Hanks' balanced salt solution (HBSS) to achieve a multiplicity of inoculum of 2 to 5 pfu per cell. Volumes of 0.2 ml and 10 ml were adsorbed for 1 hr at 36C to cell monolayers grown in 6-well plates or roller bottles, respectively. Following adsorption, the inoculum virus was removed by aspiration and replaced with various maintenance media as detailed below.

Incorporation of Labeled Precursors: BHK-21 cells were grown in BHK-21 medium (Flow Labs, Hamden, CT) with 10% fetal calf serum (FCS) to 80 to 90% confluency in 4-well plastic tissue culture plates. Immediately following infection, both infected and control cultures were treated with varying concentrations of ribavirin for 5 h at 37C and examined for their ability to incorporate labeled precursors into TCA-soluble and -insoluble pools.

Labeling of RNA and protein was accomplished by incubating cell cultures in serum-free medium containing 1 μ Ci/ml of [³H]uridine or [³H]leucine, respectively, for 30 min. Cultures were then washed 3 times with ice-cold HBSS and the radioactive content of TCA-soluble and -insoluble pools determined by scintillation spectrometry as described by Minor and Dimmock (11).

RNA Synthesis in Permeable Cells: BHK-21 cells were made permeable by treatment with lysolecithin at 4C. This procedure as well as the assay for [³H]uridine 5'-triphosphate incorporation into acid-insoluble products of permeable cells followed previously published methods (3,4).

Isolation of RNA: For the preparation of RNA, roller bottle cultures of infected and control cells were incubated in the presence of varying concentrations of ribavirin in BHK-21 media containing 2% fetal calf serum and 10 μ Ci/ml of tritiated uridine or guanosine. After 5 h, the cells were harvested, washed free of medium, resuspended at 4C in 10 mM Tris, 10 mM NaCl and 1.5 mM MgCl₂, pH 7.3 (TNM buffer) and disrupted by several freeze-thaw cycles. Cellular homogenates were

*CANONICO, LITTLE, JAHRLING,
& STEPHEN

centrifuged (1400 g for 5 min) and the nuclear pellet washed with TNM containing 1% Nonidet P-40 and 0.5% deoxycholate. Supernatants were combined and adjusted to final concentrations of 10 mM EDTA, 1% sodium dodecylsulfate (SDS) and 0.4 N sodium acetate (pH 5.2) then extracted twice with a mixture of 50% phenol, 49% chloroform and 1% isoamyl alcohol. The resulting organic phase and cake were re-extracted first with equal volumes of 0.1 M Tris (pH 9.0) in 0.5% SDS, then with 0.1 M Tris (pH 9.0). The alkaline extracts were combined with previous aqueous extracts and the RNA precipitated overnight at -20C with 2 volumes of ethanol. The precipitate was washed twice with ethanol and dried in vacuo.

For the mRNA isolation, RNA was dissolved in binding buffer consisting of 10 mM Tris (pH 7.4), 0.5 M NaCl and 0.02% SDS and applied to 0.5 g of oligo-dT) cellulose in a disposable column, then washed with 10 ml of binding buffer. Messenger RNA was eluted with 0.05% SDS in 10 mM Tris (pH 7.4), precipitated with 2 volumes of ethanol, recovered by centrifugation at 100,000 g for 1 h and dried under vacuum.

In some experiments, actinomycin D (1 µg/ml) and [³H]uridine were added to cell cultures 3.5 and 4 h, respectively, after addition of ribavirin. RNA was isolated after a 2-h incubation.

Polyacrylamide Gel Electrophoresis (PAGE): One hundred-cm 2.5% bis-acrylamide cross-linked gels were prepared as previously reported (1). The gels were prerun at 5 mA/tube and electrophoresed for 2 to 2.5 h. The RNA sample was suspended in 120 mM Tris, 60 mM sodium acetate, 3 mM EDTA (pH 7.4) plus 10% glycerol and 0.1% bromophenol blue. Following electrophoresis, gels were sliced into 1-mm fractions and incubated for 18 h at 37C with 3% Protosol in 10 ml of Econofluor (New England Nuclear) in tightly capped vials, then counted for radioactivity.

Analysis of 5'-Ends of mRNA: [³H]Guanosine-labeled mRNA was dissolved in 0.05 M sodium acetate buffer (pH 4.5) and incubated for 15 h at 37C with 2 to 5 units of ribonuclease T₂. The resulting hydrolysate was subjected to DEAE-cellulose chromatography as described by Groner and Hurwitz (8).

Cell-Free Protein Synthesis: A commercial translation assay provided by New England Nuclear, Boston, MA, was used. The assay employed reticulocyte lysate and measured incorporation of [³H]leucine.

Reagents: RNase T₂ was obtained from Sankyo Co., Tokyo, Japan. Oligo-(dT) cellulose type T-3 was bought from Collaborative Research, Waltham, MA. Reagents for casting of acrylamide gels were purchased

*CANONICO, LITTLE, JAHRLING,
& STEPHEN

from Bio-Rad, Richmond, CA. [5-³H]Uridine, [5-³H]uridine 5'-triphosphate, [8-³H]guanosine and [3,4,5-³H]leucine were purchased from New England Nuclear. Ribavirin and its mono- (RMP), di- (RDP), and tri- (RTP) phosphorylated derivatives were a gift of ICN Pharmaceuticals, Irvine, CA. Cap standards were obtained from P. L. Biochemicals, Milwaukee, WI. All other biochemicals were purchased from Sigma, St. Louis, MO.

RESULTS

Virus Replication: Replication of VEE in BHK-21 cells occurs rapidly and results in a 3-log increase in virions within 5 h after infection (Table 1). This increase is inhibited by ribavirin at concentrations as low as 10 $\mu\text{g/ml}$ by more than 99% when compared to non-treated infected cultures. Ribavirin, however, does not completely inhibit VEE virus replication. Rather it appears to reduce the rate of virus production as evidenced by the increase in virus titers following longer-term incubation of ribavirin treated infected cells.

Table 1. Effect of Ribavirin on VEE Virus Replication in BHK-21 Cells
Cells were infected with 2 pfu per cell at 37C for 1 h then incubated in BHK-21 medium with 10% FCS containing appropriate concentrations of ribavirin. Titers represent pfu/ml of supernatant culture fluid expressed as log of the reciprocal of the dilution at the endpoint of titration.

Ribavirin ($\mu\text{g/ml}$)	Log ₁₀ PFU/ml			
	Hours after virus adsorption			
	0	3	5	17
0	4.4	4.4	7.2	8.6
10	4.6	4.5	4.8	5.6
25	4.6	4.3	5.0	5.4
50	4.7	4.5	5.2	5.3
100	4.6	4.4	4.8	5.4

RNA Synthesis: The effect of ribavirin on uridine uptake and incorporation into acid-soluble material was assessed in control and virus-infected cells (Figure 1). Ribavirin inhibits uridine incorporation by more than 80% at a dose of 8 $\mu\text{g/ml}$. However, ribavirin also causes a parallel decrease in the uptake of uridine into the soluble cellular pool. Hence, the inhibition of RNA synthesis may be an arti-

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fact resulting from a decrease in the specific activity of [^3H]uridine in the soluble cellular pool of ribavirin-treated cells. RNA synthesis

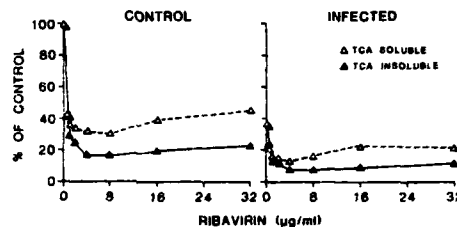


Figure 1. Effect of Ribavirin on Uptake and Incorporation of [^3H]Uridine in Control and Infected BHK-21 cells. Results are expressed as a percentage of the uptake or incorporation of [^3H]uridine found for uninfected, untreated (control) cells. Each point represents the mean value obtained from three replicate measurements.

was also assessed in infected cells made permeable to low molecular weight charged molecules by pretreatment with lysolecithin. These cells incorporated [^3H]UTP into TCA-insoluble products at a linear rate for at least 15 min. The amount of [^3H]UTP incorporation, in the presence of actinomycin D at 1 µg/ml, was not altered by ribavirin or its phosphorylated derivatives (Figure 2). These data suggest that neither ribavirin nor its phosphorylated metabolites inhibit the synthesis of mRNA in virus-infected cells.

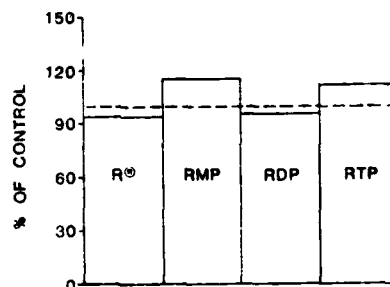


Figure 2. Effect of Ribavirin and Phosphorylated Derivatives on [^3H]UTP Incorporation in Permeable VEE-infected BHK-21 Cells. Cells were made permeable 5 h after infection for [^3H]UTP incorporation. Permeable cells were incubated for 12 min in the presence of 1 µg/ml of actinomycin D and 100 µg/ml of phosphate derivatives. Results are expressed as a percentage of the incorporation found in the absence of drug.

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The effects of ribavirin treatment on synthesis of specific mRNA was also studied. In the first series of experiments, RNA was labeled with [³H]uridine for the first 5 h following virus adsorption in the presence of 0-200 μ g/ml of ribavirin. PAGE subfractionation of oligo-(dT) cellulose binding RNA revealed a viral specific 42S mRNA at a relative migration of 0.1 and accounted for about 10 to 11% of the total mRNA isolated (Figure 3). Treatment of infected and control cultures with up to 300 μ g/ml of ribavirin did not alter the quantity and size distribution of the recovered mRNA.

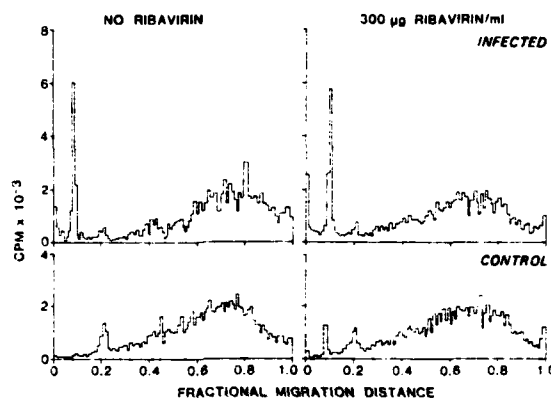


Figure 3. Polyacrylamide Gel Electrophoresis of mRNA. Infected and control cells were labeled with [³H]uridine for 5 h; then mRNA was isolated by binding to oligo-(dT) cellulose as described in Materials and Methods.

In other experiments, cells were treated with 1 μ g/ml of actinomycin D 3.5 h after infection. Thirty minutes later, [³H]uridine was added for 2 h, labeled with [³H]uridine at hour 4 to 6. Electrophoretic analysis of mRNA in these cells showed two mRNA peaks representing 42S and 26S species. Treatment of infected cells with 100 μ g/ml of ribavirin initiated 4 h prior to labeling did not later production of either mRNA species (Figure 4).

Effects on Protein Synthesis: Ribavirin at concentrations up to 32 μ g/ml, inhibited protein synthesis by 50 to 60%, as measured by the incorporation of [³H]leucine into acid-soluble products of control and infected cells. Furthermore, this reduction in leucine incorporation occurred in the presence of nearly constant levels in the soluble cellular pool of [³H]leucine (Figure 5).

The capacity of mRNA from ribavirin-treated and untreated virus-

*CANONICO, LITTLE, JAHRLING,
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infected cells to direct the synthesis of proteins in an *in vitro* cell-free translation system was evaluated in order to determine the mechanism for the inhibition of protein synthesis. Messenger RNA from

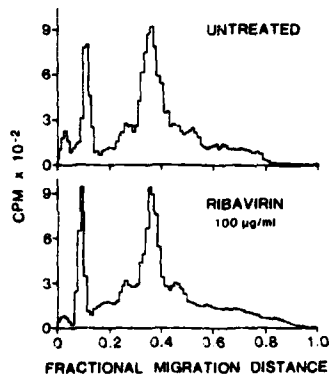


Figure 4. Polyacrylamide Gel Electrophoresis of RNA. Infected cells were pulse-labeled with [^3H]uridine for 2 h in the presence of actinomycin D (1 $\mu\text{g}/\text{ml}$) as described in Materials and Methods.

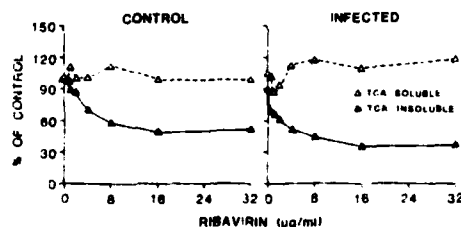


Figure 5. Effect of Ribavirin on Uptake and Incorporation of [^3H]Leucine in Control and Infected BHK-2 Cells. Results are expressed as a percentage of the uptake or incorporation of [^3H]leucine found for untreated, uninfected (control) cells. Each point represents the mean value obtained from three replicate experiments.

infected cells treated with 100 $\mu\text{g}/\text{ml}$ of ribavirin for 5 h was no more than 30% as effective as mRNA from untreated infected cells in its ability to direct the incorporation of labeled leucine into acid insoluble material (Figure 6).

Effect of Ribavirin on mRNA Cap Structure: Resulting chromatographs of [^3H]guanosine-labeled mRNA digests are shown in Figure 7. A radioactive peak corresponding to the elution profile of a cap standard

*CANONICO, LITTLE, JAHRLING,
& STEPHEN

was found in all chromatographs of mRNA digests from both control and infected cells. The magnitude of this peak decreased 3- to 10-fold in mRNA preparations from cells treated with ribavirin at concentrations of 50 to 300 $\mu\text{g}/\text{ml}$.

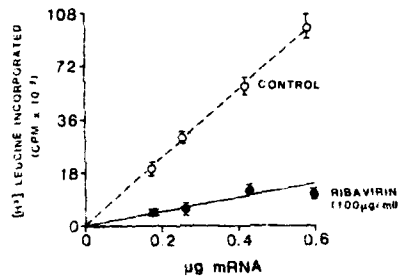


Figure 6. In Vitro Translation of mRNA in a Reticulocyte System. Incorporation of [^3H]leucine into TCA-insoluble products was measured using oligo-(dT) cellulose binding RNA from infected cells treated for 5 h with or without ribavirin.

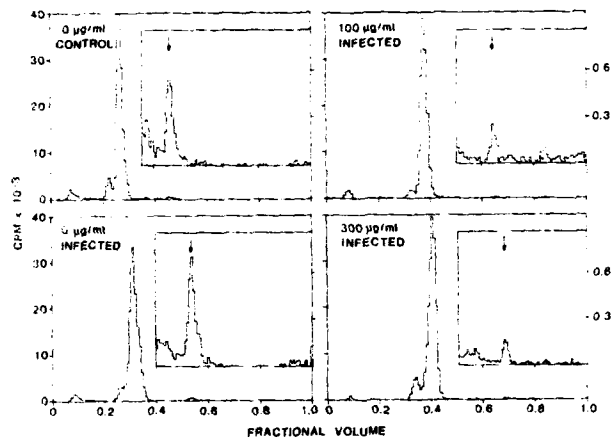


Figure 7. DEAE-Cellulose Chromatography of mRNA Hydrolysates. [^3H]Guanosine-labeled nucleotides released by RNase T₂ digestion of oligo-(dT) cellulose binding RNA. The insets in each panel represent a 1000-fold expansion of the ordinate of the corresponding chromatographs. The elution peak of a cap standard is indicated by an arrow.

DISCUSSION

Uridine incorporation has been used to assess the effect of ribavirin on RNA synthesis (2,16). Such studies have led to the proposal that ribavirin inhibits RNA synthesis. Our current finding, that the uptake of uridine by BHK-21 cells is inhibited by ribavirin, raised the possibility that the apparent inhibition of RNA synthesis by ribavirin is an artifact resulting from a decrease in the specific activity of isotopic labeling. The experiment with permeable cells, in fact, demonstrates that ribavirin does not affect actinomycin D-insensitive RNA synthesis in BHK-21 cells. Furthermore, the quantity of mRNA isolated by oligo-(dT) cellulose binding showed that ribavirin has little if any effect on the total amount of mRNA in either control or virus-infected cells.

The inhibition of protein synthesis in both control and infected cells exposed to varying concentrations of ribavirin cannot be explained by reduced levels of mRNA in these cells. The inefficiency in translation of mRNA from ribavirin-treated cells suggests that treatment with ribavirin results in synthesis of altered mRNA.

Recently, in vitro studies by Goswami et al. (7) showed that ribavirin triphosphate is a potent competitive inhibitor of the capping guanylation of viral mRNA. This posttranscriptional modification results in a 7-methylguanosine residue linked from its 5' position via a triphosphate bridge to a 2'-O-methylribonucleotide of the 5'-terminal of a large number of viral and eukaryotic mRNA. The 5'-terminal 7-methylguanosine in mRNA is required for efficient translation (13). The present data show that ribavirin also inhibits the formation of the 5'-guanosine triphosphate cap on mRNA from normal and virus-infected BHK-21 cells. Interference with the proper formation of the 5'-cap of mRNA by ribavirin could lead to accumulation of mRNA in cells which are, as observed, less efficient in protein synthesis.

The cap structure analysis was performed on digests of mRNA labeled with guanosine for 5 h following virus adsorption and addition of varying concentrations of ribavirin. This was done in order to obtain the largest representation of mRNA species which may have contributed to the impairment of protein synthesis in ribavirin-treated infected cells. Under these conditions about 90% of the radiolabeled oligo-(dT) binding RNA from infected cells is of host cell origin. Hence, host cellular mRNA must be largely responsible for the 3- to 10-fold reduction in cap formation of virus-infected cells. It is clear that ribavirin's effect on cap formation is nonspecific and leads to inhibition of both cellular and viral protein synthesis. Consistent with previous observations, ribavirin can be expected,

*CANONICO, LITTLE, JAHRLING,
& STEPHEN

therefore, to interfere with virus as well as cellular growth (16).

Although Goswami *et al.* (7) proposed that inhibition of cap formation may account for ribavirin's antiviral potency against DNA and RNA viruses, it is unclear to what extent other mechanisms may play a role. It is apparent that ribavirin exerts a myriad of effects on cellular metabolism and it is conceivable that its antiviral effects may be, in fact, expressed through an interaction of multiple mechanisms.

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*CANONICO, LITTLE, JAHRLING,
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