TABLE IV. Simultaneous Titrations of R.S. Virus Positive Original Specimens in WI-38 and HEp-2 Cell Cultures.

| Specimen | Titer* WI-38 | Titer* HEp-2 |
|---|---|---|
| 6206A | 1.50 | 1.20 |
| 6249A | 1.20 | .80 |
| 6300A | 2.20 | 1.20 |
| 5933-1.23 | 2.50 | .80 |
| 6184B | undiluted | negative |

* $TCID_{50}$/0.1 ml expressed as recip. $\log_{10}$.

R. S. virus. Although initial manifestations of viral growth were evident somewhat earlier in HEp-2 than in diploid cells, isolation rates in the two types of cell cultures were not significantly different. Comparative infectivity titrations of original patient specimens in HEp-2 and WI-38 cell cultures suggested the possibility of a slight advantage in sensitivity for the latter cell.

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## Reversible Inhibition of DNA Virus Replication with Mithramycin.* (30739)

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Mithramycin, an antibiotic derived from cultures of Streptomyces, has been the subject of many studies as an investigational therapeutic agent in the treatment of cancer(1-5). Although the drug has a rather narrow range of therapeutic efficacy, principally limited to embryonal neoplasms, and a characteristic toxicological pattern(6), little is known of the mechanism of action resulting in cell death. This report deals with the effects of mithramycin on viral replication and shows that the antibiotic inhibits the synthesis of DNA but not RNA viruses.

*Materials and methods.* The effects of mithramycin† on several viruses in various tissue culture systems were studied (Table I). Mouse embryo fibroblasts obtained by trypsinizing 10-12-day Swiss mouse embryos were grown in one-liter Blake bottles in Eagle's medium (BME)(7) containing 100 units/ml penicillin, 100 μg/ml Streptomycin and 100 μg/ml Fungizone and supplemented with 5% calf serum. Rabbit kidney cells prepared in a similar fashion from the renal cortex of 2 kg albino rabbits were grown in BME containing 10% horse serum. "HeLa" and "L" cells were maintained in Blake bottles in BME with 5% calf serum and subcultured every 10-12 days. For inhibition experiments, cells were used within 24 hours of subculturing. Cell monolayers in 60 mm plastic petri dishes were pretreated with mithramycin in BME for 4 hours prior to virus inoculation. Following virus absorption the cells were washed in Hanks' balanced salt solution and then overlaid with medium containing the inhibitor. After one or two virus growth cycles, the cultures were frozen, thawed and virus yields determined by titration using the plaque method(8). The yields with mithramycin were compared with titers in paired control cultures. All cultures were incubated at 37°C in a humid atmosphere at 5% $CO_2$, 95% air.

Initial experiments to determine the susceptibility of the different cell types to the

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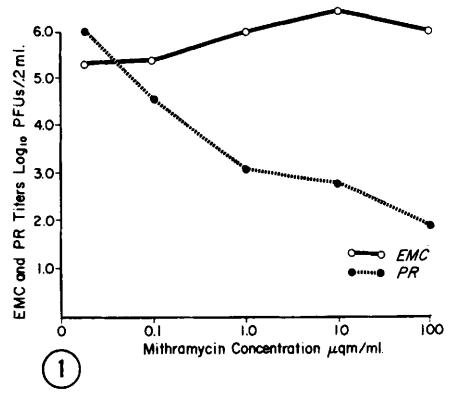


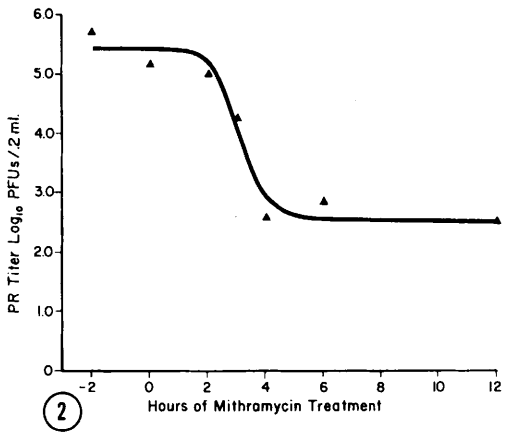


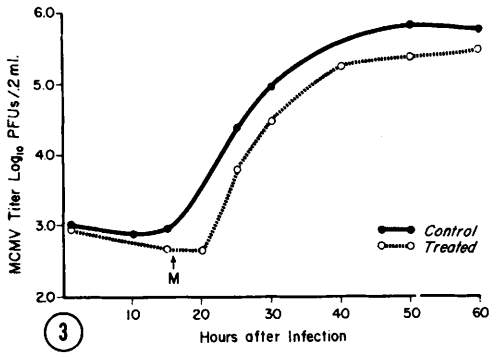


FIG. 1. Dose response curve of pseudorabies (PR) inhibition compared to yields of encephalomyocarditis virus (EMC) at the same concentrations of mithramycin. Plaque titrations were done 12 hr after inoculation of cultures.

FIG. 2. Reversal of mithramycin inhibition of pseudorabies (PR) virus. Cell cultures were pretreated with mithramycin for 2 hr prior to inoculation. At intervals following infection, the mithramycin medium was removed, the cultures washed and fresh inhibitor-free medium was added. All cultures were frozen and titrated at 12 hours.

toxic effect of mithramycin were carried out in triplicate cultures. After 24 and 48 hours' exposure to the drug, cell monolayers were vitally stained with neutral red, graded for degeneration and the $LD_{50}$ calculated by the Reed-Muench method(9).

*Results.* The $LD_{50}$ of mithramycin for "L" and "HeLa" cells was approximately 50 μg/ml; for mouse embryonic fibroblasts, 10 μg/ml. At concentrations of 2 μg/ml all cell types tested remained capable of supporting virus replication for periods up to 48 hours.

Mithramycin at concentrations of 100 μg/ml had no inhibitory effect on the two RNA viruses, polio in "HeLa" cells and encephalomyocarditis (EMC) in "L" and rabbit kidney cells (Table I). In contrast, the yields of 2 DNA viruses, pseudorabies (PR) and murine cytomegalovirus (MCMV) were markedly reduced at levels of 10 and 1 μg/ml respectively. A dose response curve with both EMC and PR is shown in Fig. 1. Significant inhibition of PR occurred at a concentration of 0.1 μg/ml with maximum inhibition at approximately 10 μg/ml. Of some interest was the persistent elevation in EMC yields in mithramycin-treated cultures as compared to control cultures. This increment in EMC titer with mithramycin is probably the result of inhibition of interferon synthesis.

In an attempt to define the mechanism of PR and MCMV inhibition, thymidine, cytidine and excess glutamine were added to the mithramycin medium with no effect on inhibition. However, as shown in Fig. 2, inhibition was prevented by removing mithramycin early in the growth cycle. When the drug was washed out prior to 3 hours following PR infection, no inhibition was obtained. Whereas, washing after 4 hours could not prevent the mithramycin effect and inhibition was maximum. Similar results were obtained with MCMV with complete reversal of inhibition on removing the antibiotic prior

FIG. 3. Late emergence of new murine cytomegalovirus (MCMV) following 16 hours of mithramycin treatment. At point "M" the mithramycin medium was removed, the cultures washed and fresh inhibitor-free medium was added.

TABLE I. Virus Yields of 2 RNA* and 2 DNA† Viruses in Various Tissue Culture Systems With and Without Mithramycin.

| Virus | Cell | Control medium titer (PFUs)‡ | Mithramycin medium titer (PFUs)‡ | Mithramycin concentration ($\mu$g/ml) |
|---|---|---|---|---|
| Poliomyelitis* | "HeLa" | $6 \times 10^7$ | $2 \times 10^7$ | 100 |
| Encephalomyocarditis* | "L" | $2 \times 10^8$ | $3 \times 10^8$ | 100 |
| " * | Rabbit kidney | $6 \times 10^6$ | $3 \times 10^6$ | 10 |
| Pseudorabies† | " " | $3 \times 10^6$ | $1 \times 10^2$ | 10 |
| Murine cytomegalovirus† | Mouse embryo | $6 \times 10^5$ | $9 \times 10^2$ | 1 |

‡ PFUs: Plaque forming units/0.2 ml.

to the first 5 hours of the 24-hour growth cycle. With removal of mithramycin after the period of reversibility during one cycle of virus multiplication, there was late emergence of infectious virus, as illustrated in Fig. 3.

Yarbro(10) has shown that mithramycin prevents the incorporation of $P^{32}$ into RNA of mouse ascites tumor cells and concluded that the antibiotic inhibits the synthesis of RNA with an effect similar to actinomycin-D. Many RNA viruses including polio and EMC are unaffected by actinomycin-D presumably because the viral RNA codes for the synthesis of new viral nucleic acid and protein without a DNA-dependent intermediary (11). Our data concerning viral inhibition with mithramycin are, therefore, in agreement with Yarbro's conclusion and, as with actinomycin-D, focus particular attention to viral DNA-directed RNA synthesis as the level of virus inhibition. Unlike inhibition by actinomycin-D which is irreversible(12), this inhibition appears fully reversible.

*Summary.* Mithramycin in concentrations of 1.0-100 $\mu$g/ml prevented the multiplication of the DNA viruses, pseudorabies and murine cytomegalovirus, but not the RNA viruses, encephalomyocarditis and polio, in tissue culture. The inhibition was fully reversible by removing the mithramycin early in the virus growth cycle. With removal of the drug at a later state, there was delayed emergence of new virus. The data suggest that mithramycin reversibly inhibits the synthesis of viral DNA-directed RNA.

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