Table 2. Effect of a single oral dose (250 mg/kg) of tilorone hydrochloride on encephalomyocarditis infections in mice. Mice were inoculated subcutaneously with 8 LD_{50} of virus (lethal dose for 50 percent of the mice). Survivors were determined 10 days after infection.

Treatment time (hr)	Day of death (mean)	Survivors (%)
	Before infection	
48	8.2	40
24	10.6	90
3	5.7	0
	After infection	
3	5.6	0
24	4.0	0
48	4.5	0
	Infected controls	
	4.4	0

as indicated by an increase in the mean day of death. Tilorone given 24 or 48 hours after infection was inactive.

Groups of 20 mice were treated orally with tilorone to determine the optimum time of treatment against Semliki Forest virus. Mice treated with 125 mg/kg 72, 48, 24, or 2 hours before subcutaneous virus challenge (34 LD_{50}, lethal dose of virus which kills 50 percent of animals) had 15 percent, 40 percent, 100 percent, and 20 percent survivors, respectively. Mice treated with 250 mg/kg at 96, 72, 48, 24, or 6 hours before intracranial virus challenge (24 LD_{50}) had 20 percent, 40 percent, 80 percent, 40 percent, and 0 percent survivors, respectively. Thus, the optimum time of treatment depends on the route of virus challenge.

A single oral dose of 100 mg/kg of tilorone, given 24 hours before infection, totally protected male CFE rats (170 g) from paralysis by subcutaneous inoculations of Semliki Forest virus, whereas 70 percent of infected controls were affected.

The LD_{50} for a single dose orally administered to mice is 959 mg/kg, and intraperitoneally administered it is 145 mg/kg. In rats the LD_{50} for a single oral dose is 852 mg/kg, and for a single intraperitoneal dose it is 244 mg/kg.

Tilorone hydrochloride has the broadest spectrum of any orally active antiviral agent yet reported.

RUSSELL F. KRUEGER
GERALD D. MAYER

Department of Microbiology,
The Wm. S. Merrell Company,
Division of Richardson-Merrell Inc.,
Cincinnati, Ohio 45215

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2. Tilorone hydrochloride was first referred to as bis-DEAE-fluorenone.
3. We thank S. Yoshimura for technical assistance.

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Tilorone Hydrochloride: Mode of Action

Abstract. *An antiviral serum component is found in mice treated orally with tilorone hydrochloride. The active material fulfills sufficient biological criteria to be classified as an interferon.*

Tilorone hydrochloride is a broad-spectrum antiviral agent active in vivo (*1*). Oral administration stimulates mice to make a protein with the biologic characteristics of interferon.

The compound, dissolved in aqueous 0.15 percent hydroxyethyl cellulose, was administered by gavage to CF-1 male mice weighing 15 to 18 g. To determine the antiviral activity of the serum samples collected by orbital bleeding, pooled serum from groups of at least ten mice was diluted in Eagle's basal medium containing Earle's salts (EBME) to which fetal calf serum (2 percent) and sodium bicarbonate (5 percent) were added. One milliliter of diluted test serum was added to each of six test tubes that contained confluent monolayers of mouse L929 cells. After incubation at 37°C for 24 hours, the serum was removed and the cells were washed twice with Hanks balanced salt solution. One milliliter of vesicular stomatitis virus (VSV) preparation, diluted in EBME to the challenge concentrations, was added to four of the six tubes; the remaining two tubes received only 1 ml of EBME and served as controls. After a 2-hour adsorption period, the cells were washed once with the Hanks solution, and 1 ml of EBME was added. The protective serum dilution (PSD_{50}) represents the dilution of serum that protected 50 percent of the cells from viral cytopathology after 48 hours. Unless stated otherwise, this was the method of assay. Active serum used for interferon characterization was collected from mice 20 to 30 hours after oral treatment with tilorone (250 mg/kg).

Table 1. Effect of enzyme exposure, dialysis, and low *p*H on active mouse serum. Serum titrations were conducted against vesicular stomatitis virus in mouse L929 cells with 50 percent cytopathology as the endpoint.

Treatment	Time of exposure	Serum titer
Trypsin	24 hr, 37°C	$<1:50$
No trypsin		1 : 3200
Ribonuclease	20 hr, 37°C	1 : 800
No ribonuclease		1 : 800
Dialyzed	24 hr	$>1:2500$
Not dialyzed		$>1:2500$
Acidified to *p*H 2.0	24 hr, 4°C	1 : 2560
Not acidified		1 : 2560

The logarithmic curve of the reciprocal of the serum dilution shows that antiviral activity appears as early as 12 hours, reaches a peak at 24 hours, and essentially disappears within 48 hours after oral treatment (Fig. 1).

Serum collected from mice 24 hours after oral treatment with different concentrations of tilorone was titrated on mouse L929 cells against 32 TCD_{50} (tissue culture dose of virus that destroys 50 percent of cells) of vesicular stomatitis virus. A dose response relative to detectable antiviral activity of mouse serum was observed (Fig. 2). The PSD_{50} ranged from a dilution of 1:15 with a dose of tilorone of 5 mg/kg to more than 1:10,000 with a dose of 250 mg/kg. A plateau of stimulation was reached between 250 and 500 mg/kg.

A 1:20 dilution of active serum, that completely protected mouse L929 cells from the cytopathology caused by encephalomyocarditis virus and vesicular stomatitis virus, failed to protect human epithelial (HEp-2) or primary rat embryo cells from the cytopathic effects of these viruses. The cytopathic effects caused by 32,000 TCD_{50} of yellow fever virus in mouse L929 cells were also prevented by this serum.

The effects of enzymes, dialysis, and low *p*H on active mouse serums are seen in Table 1. A 10 percent solution of active mouse serum diluted in EBME was incubated with 0.01 percent trypsin for 24 hours at 37°C. After soybean trypsin inhibitor was added (0.02 percent final concentration), the antiviral activity of the trypsin-digested serum was compared with that of undigested serum containing the soybean inhibitor. The PSD_{50} of the digested serum was less than 1:50 (lower dilutions not tested), whereas that of the undigested serum was 1:2400. Trypsin digestion at 37°C for 1 hour destroyed 95 percent of the antiviral potential of active mouse serum. Exposure of active mouse se-

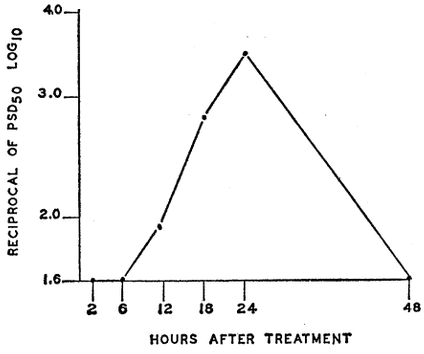

Fig. 1. Appearance of antiviral activity in mouse serum after oral administration of tilorone hydrochloride (250 mg/kg). Activity was measured on mouse L929 cells against vesicular stomatitis virus. First dilution of serum was 1:50.

rum to 0.01 percent ribonuclease for 20 hours at 37°C did not alter its activity. Dialysis of active serum against distilled water at room temperature did not remove the antiviral component. Active mouse serum, diluted in EBME and acidified to *p*H 2.0 with 0.1*N* hydrochloric acid was refrigerated for 24 hours at 4°C. The acid-treated serum, neutralized by dilution in sodium bicarbonate–buffered EBME, retained its activity.

Active mouse serum was compared with serum from untreated mice for inactivation of encephalomyocarditis virus and vesicular stomatitis virus. One to ten dilutions of both serums were prepared in an EBME suspension of each virus. The serum-virus suspensions were incubated for 3 hours at 37°C after which dilutions were made in EBME and applied to monolayers of rabbit kidney cells (RK_{13}). The titer of vesicular stomatitis virus incubated with normal serum was $10^{5.8}$ TCD_{50}/ml and its titer when incubated with active serum was $10^{6.0}$ TCD_{50}/ml. The titer of encephalomyocarditis virus incubated with normal serum was $10^{5.5}$ TCD_{50}/ml and its titer when incubated with active serum was $10^{5.4}$ TCD_{50}/ml. Thus, there was no evidence of virus inactivation by active serum.

If the antiviral component in the serum of mice treated with tilorone is interferon, then serum activity observed in the homologous, mouse L929 cell line should be dependent on the synthesis of new messenger RNA (*2*). A 1:40 dilution of serum with a PSD_{50} of 1:3000, collected from mice treated orally, was incubated for 4 hours at 37°C on confluent monolayers of mouse L929 cells (12 tubes) in the presence of 1.0 μg of actinomycin D per milliliter. After incubation, the cells were washed three times with Hanks balanced salt solution and challenged with 30,000 TCD_{50} of vesicular stomatitis virus. After 1 hour, the cells were washed four times with the Hanks solution to remove extracellular virus; 1.0 ml of EBME was added, and the tubes were incubated again for 20 hours. Groups (12 tubes) of normal serum, normal serum plus actinomycin D, and active serum without actinomycin D were treated similarly. The contents of all tubes within a group were pooled, and virus was titrated by plaque assay. Plaque assay was accomplished by the addition of dilutions (0.2 ml) of virus to confluent monolayers of monkey kidney cells ($LLC\text{-}MK_2$) grown in disposable plastic petri dishes (60 mm by 15 mm; Falcon). After a 2-hour adsorption period at 37°C the cells were washed three times with the Hanks solution to remove extracellular virus. An overlay of a mixture of 0.5 percent methylcellulose and EBME was applied to the monolayers which were incubated at 37°C. Seventy-two hours later, the overlay was removed, the cells were stained with 0.2 percent crystal violet in deionized water, and plaques were counted to determine virus yield. Actinomycin D significantly inhibited the protection normally provided by serum from mice treated orally with tilorone. The virus yield from cells treated with normal mouse serum was $10^{8.1}$ plaque-forming units (PFU) per milliliter, while cells treated with active mouse serum yielded $10^{6.0}$ PFU/ml. The virus yield from cells treated with normal mouse serum in the presence of actinomycin D was $10^{7.6}$ PFU/ml. The virus titer from cells treated with actinomycin D and active serum was $10^{7.5}$ PFU/ml.

We conclude that the antiviral component found in mice treated orally with tilorone is interferon because it (i) is species specific, (ii) has broad antiviral activity, (iii) is inactivated by exposure to a proteolytic enzyme, (iv) is resistant to ribonuclease, (v) is not dialyzable, (vi) is stable to low *p*H, (vii) is not virucidal, and (viii) requires new messenger RNA synthesis for activity as indicated by sensitivity to actinomycin D. Tilorone hydrochloride represents the first recognized synthetic, small molecular weight compound that is an orally active inducer of interferon.

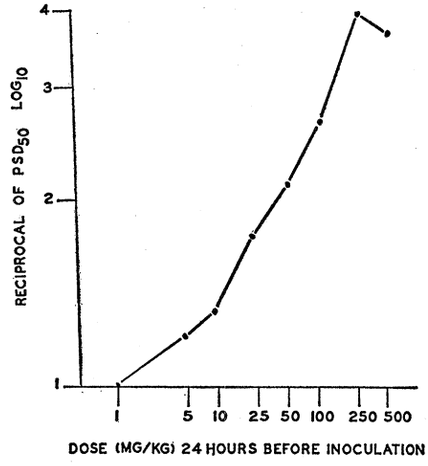

Fig. 2. Quantitative relation of tilorone dose to stimulation of the antiviral serum component in mice. Tilorone was given orally 24 hours before serum collection. Antiviral activity measured against vesicular stomatitis virus (32 TCD_{50}) in mouse L929 cells.

GERALD D. MAYER
RUSSELL F. KRUEGER
Department of Microbiology, The Wm. S. Merrell Company, Division of Richardson-Merrell Inc., Cincinnati, Ohio 45215

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Enzyme Inactivation with Ultraviolet Laser Energy (2650 Angstroms)

Abstract. *Inactivation of rat heart lactate dehydrogenase was accomplished by irradiation of the enzyme in solution with a frequency quadrupled neodymium glass laser.*

Laser energy has shown biological and biochemical activity in the visible region of the spectrum. Due to the fact that only a few natural chromophores are available for absorption of the visible wavelengths, the biological effectiveness of lasers has been limited to studies that made use of these