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DEVELOPMENT OF SYSTEMS FOR DELIVERY OF ANTIVIRAL DRUGS

FINAL REPORT

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October 1989
(For the period 30 September 1985 - 14 September 1989)

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This is our Final Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period September 30, 1985 through September 14, 1989.

Ribavirin, a broad-spectrum antiviral agent with potent activity *in vitro* against a number of important RNA viruses of military significance, is severely limited in its usefulness against virus-induced encephalitic diseases because it does not cross the blood-brain barrier well enough to achieve effective antiviral concentrations in the brain. Our efforts have been directed toward the brain-specific delivery of ribavirin and other antiviral agents by means of a redox prodrug concept. The scope of the research program involves the synthesis of CNS-targeted prodrug esters of ribavirin and selenazole, pharmacokinetic studies of drug distribution and sustained delivery of drug in the brain, and the evaluation of the therapeutic efficacy of these antiviral prodrugs compared with the parent drugs in the treatment of lethal Venezuelan equine encephalitis (VEE) virus, Japanese encephalitis (JE) virus, and Punto Toro PT) virus infections in mice. In preliminary studies at USAMRIID, the initial ribavirin prodrug protected mice from a lethal challenge of JE virus and was much superior in efficacy to the parent drug which had

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no effect. Initial attempts to confirm the original observation of *in vivo* efficacy of this ribavirin prodrug at Southern Research Institute were unsuccessful, but differences in virus strain, route of inoculation, and treatment protocol may have accounted for the failure to detect antiviral efficacy. *In vivo* antiviral evaluations were begun with the ribavirin prodrugs in CD-1 Swiss mice infected with JE (Nakayama strain) or VEE (Trinidad donkey strain). Therapeutic efficacy was not observed with either ribavirin or the initial prodrug in these two models under the conditions of assay. The JE virus challenge was administered by the intracranial route, while the VEE virus was administered i.p. These studies have been repeated with the Beijing strain of JE virus administered i.p. to C57B1/6 mice and using a chemoprophylaxis protocol as employed in the earlier studies at Ft. Detrick. Increases in the mean survival time of drug-treated animals were noted. Additional ribavirin prodrugs have been synthesized and evaluated in the JE model. While no reduction in mortality was observed, increases in the mean survival time of drug-treated animals were observed. Higher dose levels or prolonged therapy might be even more effective. An intracranial challenge model with Punta Toro virus was developed and target organ therapy protocols were designed for the evaluation of ribavirin and ribavirin prodrugs. Unfortunately, the contract period terminated before these latter models could be utilized for antiviral drug testing *in vivo*.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1986).

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I. INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been found to possess broad-spectrum antiviral activity both *in vitro* and *in vivo* (1-5). Studies conducted at Fort Detrick have clearly demonstrated that ribavirin is markedly effective against bunyaviruses (e.g., Rift Valley fever virus) and arenaviruses (e.g., Lassa fever virus, Pichinde virus, and Machupo virus), but that it has only minimal to no efficacy against the alphaviruses (e.g., Venezuelan equine encephalitis virus and Chikungunya virus) or flaviviruses (e.g., Japanese encephalitis virus and yellow fever virus) *in vivo*. The apparent inability of ribavirin to achieve effective antiviral concentrations in the brain and central nervous system significantly limits its usefulness against those viruses which cause primary encephalitis (6). Ribavirin also does not prevent the late encephalitic phase of the diseases caused by Rift Valley fever, Junin, and Machupo viruses (7). The principal reason for this lack of efficacy is the relative inability of the drug to cross the blood-brain barrier and to concentrate in the central nervous system.

A related compound (2-β-D-ribofuranosylselenazole-4-carboxamide; selenazole) has been synthesized by Srivastava and Robins (8) and has been shown to exhibit potent, broad-spectrum *in vivo* activity against selected DNA-containing and RNA-containing viruses (9), including many viruses of potential military importance. Selenazole appears to be significantly more potent than ribavirin against paramyxoviruses, reoviruses, togaviruses, bunyaviruses, arenaviruses, picornaviruses, rhabdoviruses, herpesviruses, and pox virus *in vitro*. It is extremely active against yellow fever virus and is a prime candidate for antiviral chemotherapy studies *in vivo*. Another compound, tiazofurin, has been found to exert *in vitro* activity against flaviviruses and Korean hemorrhagic fever (KHF) virus. Both selenazole and tiazofurin appear to be rapidly excreted *in vivo*, so that development of a prodrug form of these drugs would be desirable.

When selenazole is used in combination with ribavirin *in vitro*, synergistic antiviral effects are observed against Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus (10). Synergistic activity has been shown for tiazofurin in combination with ribavirin against yellow fever virus *in vitro*, but not for other viruses. These observations indicate that combination antiviral chemotherapy with these agents and ribavirin might be a useful approach to the treatment of flavivirus infections *in vivo*.

Since many of the target viruses of interest to the Army produce a lethal encephalitis in the host, we believe that efforts directed toward the brain-specific delivery of candidate antiviral drugs will be an important approach to improving the efficacy of such drugs against agents of military significance.

Our initial efforts were directed toward the synthesis and evaluation of ribavirin prodrugs. Based upon the brain-specific delivery of, for example, phenethylamine (11), dopamine (12), trifluorothymidine (13), and acyclovir (14), we expected prodrug esters of ribavirin to effectively cross the blood-brain barrier. Once in the brain, the dihydropyridine moiety would be expected to be oxidized to the pyridinium salt which would be retained. Cleavage of the ester enzymatically would then produce a sustained delivery of ribavirin in the brain. For any specific compound, the rates of the various reactions in the process must be favorable, but the success achieved in several systems thus far certainly give credence to our proposed application of this redox delivery system approach to the sustained, site-specific administration of antiviral agents such as ribavirin.

The early results obtained with our initial ribavirin prodrug (see First Annual report, dated October 31, 1986), were encouraging in that the compound protected mice from a lethal challenge with JE virus when administered by the i.p. route.

During the second year, we concentrated on developing pharmacological data on the metabolism and disposition of our initial ribavirin prodrug in mice, on the synthesis of new ribavirin prodrugs that are cleaved either more rapidly or more slowly than the original prodrug that was synthesized during the first year of this contract, and on the development of our *in vivo* antiviral evaluation systems involving lethal JE virus and VEE virus infections in mice. The initial ribavirin prodrug was evaluated in these animal infection models and the results compared with those obtained earlier at Fort Detrick. While the initial results obtained at USAMRIID could not be confirmed, useful information was obtained with regard to the importance of the virus strain,

route of inoculation, and the test protocol for additional antiviral evaluations in our laboratories at Southern Research Institute.

During the third year, further efforts were directed at the synthesis of additional prodrugs of ribavirin and we successfully completed the synthesis of the original prodrug form of selenazole in quantities sufficient for preliminary antiviral evaluation. The Beijing strain of JE virus was obtained and passaged in C57Bl/6 mice numerous times until the stock virus preparations produced 100% mortality in inoculated animals. Large stocks of challenge virus were prepared and titered *in vivo*. Antiviral chemotherapy experiments were conducted to evaluate three compounds (SRI-6711, SRI-7222, and SRI-7223) in this model system. While no reduction in mortality was observed, there were increases in the mean survival time of drug-treated mice compared with controls. Results of these experiments indicated that either higher dose levels or prolonged therapy might be even more effective.

During the last year, work was initiated on development of animal models relevant to the research program. Two approaches were used. First, an animal model which is sensitive to the antiviral effects of ribavirin was pursued. This model is based on the differential lethality of the Balliet strain of Punta Toro (PT) virus. This virus is lethal when the challenge route is intracranal; while peripheral challenge is nonlethal. Thus, compounds with anti-PT activity would potentially be active if they crossed the blood-brain barrier.

Secondly, target organ systems were under development such that small quantities of compound could be tested for *in vivo* efficacy prior to synthesis of bulk drug quantities. These systems are similar to those developed for herpes and vaccinia viruses (13,14). The virus inoculum is administered into the target organ. This is followed by administration of a single dose of compound into the target organ. Studies with VEE and JE were conducted to define the intracerebral (i.c.) LD_{90} challenge levels. Following definition of the challenge dose, known *in vivo* active compounds could be tested for activity in the target organ system if desired by USAMRIID.

II. CHEMISTRY

A. Introduction

Our efforts during the tenure of this contract resulted in preparative prodrug 1 by two synthetic methods, the second method being the method of choice in providing larger quantities of this prodrug.

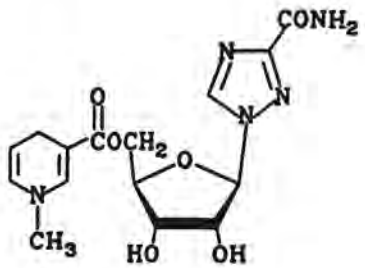
Because desired pharmacokinetics may depend on the facility of ester cleavage we prepared compound 13. Reduction of the steric bulk on the pyridine side of the ester linkage by addition of a methylene unit would provide for a faster release of ribavirin.

We prepared radiolabeled 1 for biological evaluations.

We achieved the synthesis of a prodrug form of selenazofurin.

B. Research Accomplished

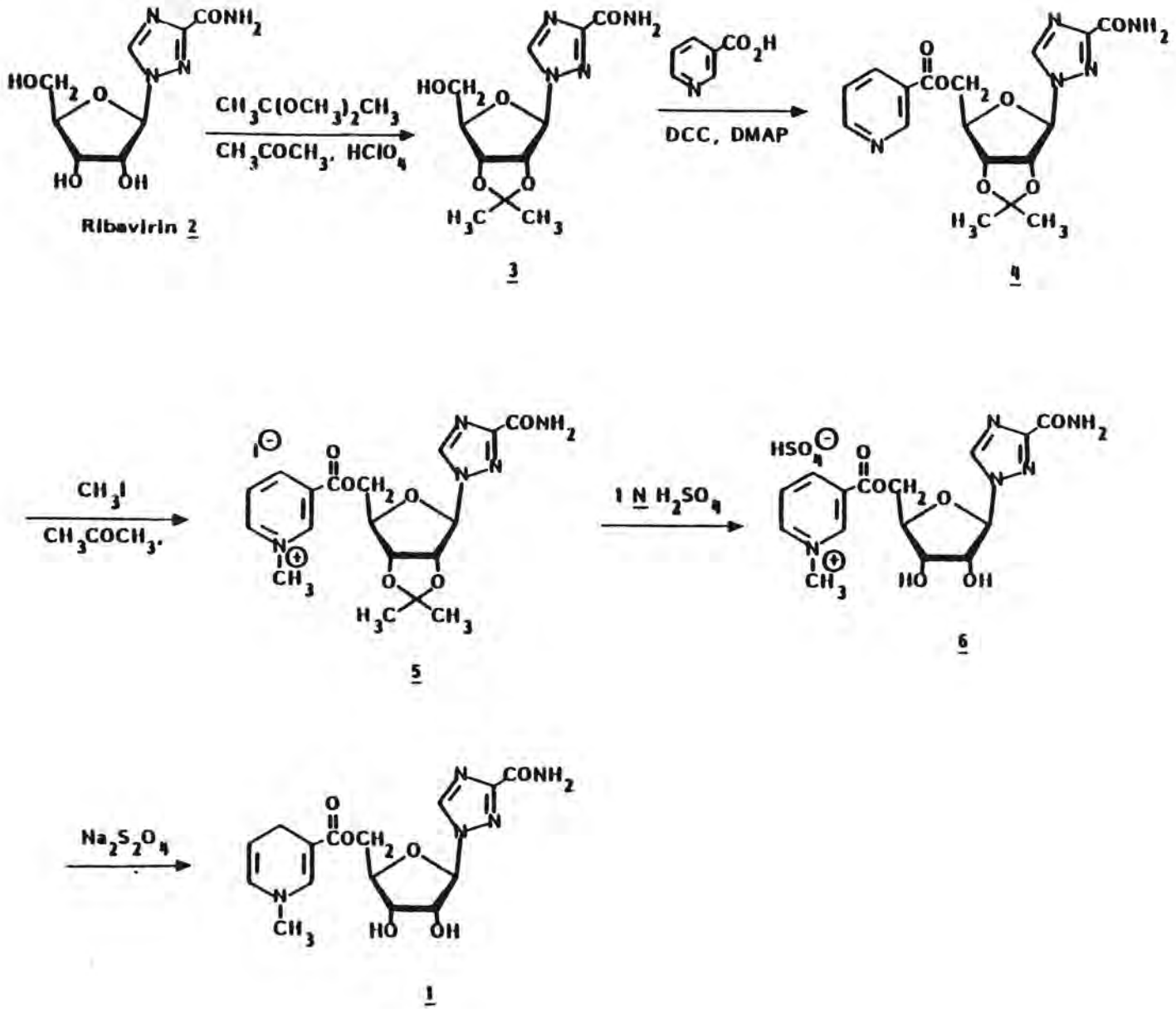
We developed a synthesis of the ribavirin prodrug 1, starting from ribavirin. This prodrug is expected to be transported across the blood brain barrier, and then rapidly oxidized from the dihydropyridine to the pyridinium salt, which will be trapped in the brain. Esterase hydrolysis of the prodrug would then gradually free ribavirin.



1

Our original synthesis of 1 is shown in Scheme I. We used dicyclohexylcarbodiimide to couple 3 to nicotinic acid. This step necessitated protection of the 2′,3′-hydroxyl groups of 1 in order to cleanly form the 5′-nicotinate ester of ribavirin. The acid-labile isopropylidene group appeared to be an appropriate candidate for a blocking group and the 2′,3′-*O*-isopropylidene derivative of ribavirin 3 is a known compound (15). Preparation of 3 was accomplished by the literature procedure, but using a flash chromatography purification. Coupling of 3 to nicotinic acid was achieved utilizing dicyclohexylcarbodiimide to produce the ester 4. Methylation of 4 on the pyridine nitrogen was accomplished with iodomethane in boiling acetone to give 5. That methylation did not occur on the triazole ring was clear from the mass spectral fragmentation pattern. Removal of the isopropylidene group from 5 occurred readily with 1*N* H_2SO_4 at room temperature to afford 6. Reduction of 6 to the target prodrug 1 was achievable with sodium dithionite under carefully controlled conditions. The reaction is best followed by HPLC, because some oxidation of 1 back to 6 occurs on a standard thin-layer plate. Proof of this oxidation on the plate was obtained by the two-dimensional development of a plate spotted with 1.

Scheme 1

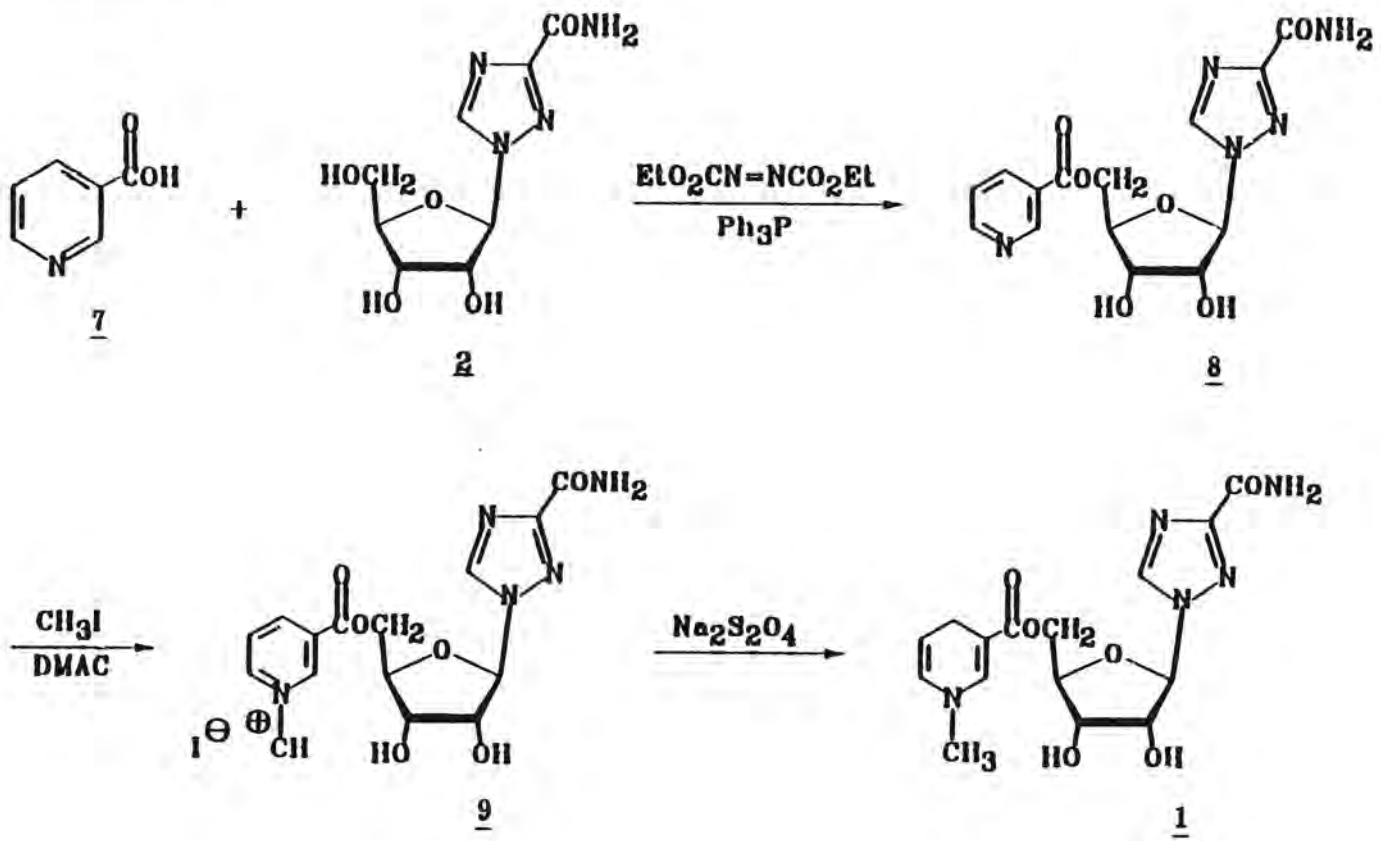


Our current route to 1 is shown in Scheme 2 and is somewhat shortened from the one that we originally used. Conducting the coupling of the acid and the ester with triphenylphosphine and diethyl azodicarboxylate eliminates the necessity of blocking the 2′- and 3′-hydroxyls with the isopropylidene group.

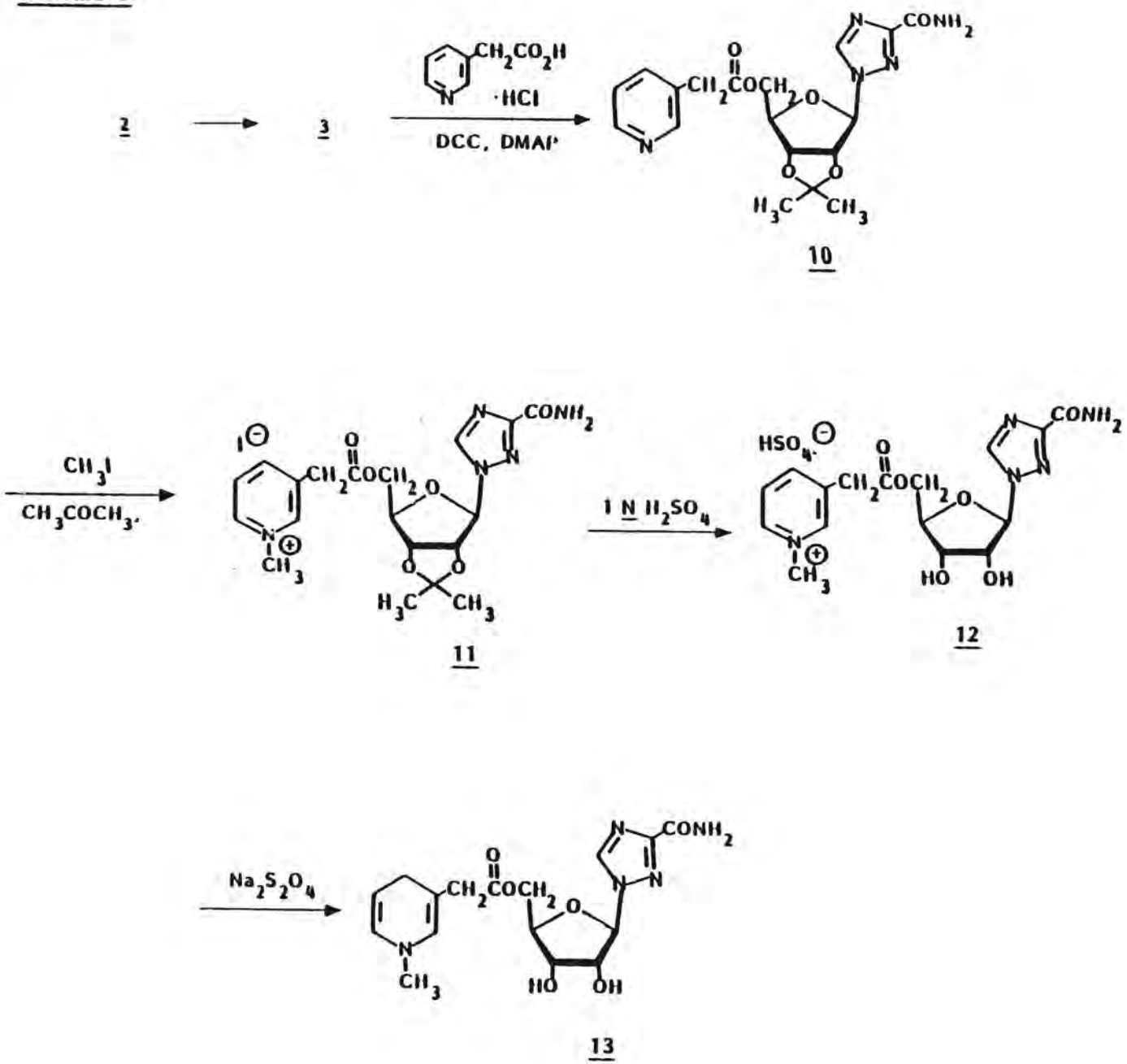
A potential problem with 1 is that once it is trapped in the brain, it may not release ribavirin as rapidly as desired. This process would depend upon the facility of ester cleavage. The aromatic ring directly adjacent to the ester linkage slows down the cleavage for steric reasons. Bulk on either side of the ester linkage will have such an effect. If the desired pharmacokinetics are to be obtained by speeding up esterase cleavage, then reducing the bulk on the pyridine side of the ester linkage should have the appropriate effect. A reduction in the steric bulk in the immediate area should be readily accomplished simply by adding a methylene unit. We therefore began efforts to synthesize the prodrug **13** by the route shown in Scheme III. The isopropylidene derivative of ribavirin (3) is coupled with 3-pyridylacetic acid in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine to produce ester **10**. Quaternization with iodomethane was carried out following the procedure employed for **5** and deblocking of the 2′,3′-hydroxyls was achieved by treatment with 1*N* H_2SO_4. Reduction of **12** proved difficult because we found that whether we used the standard reducing agent sodium dithionite or various other reducing agents such as sodium borohydride and sodium cyanoborohydride, we could not effectively stop the reduction at the dihydropyridine state. Proper experimental conditions do allow the isolation of a tetrahydro compound. When the 2′,3′-unblocked compound **12** was treated with sodium borohydride in anhydrous DMF, compounds **14-17** were formed. These prodrugs are shown in Scheme 3A. We were unsuccessful in our attempts to cleave the B-O bonds of compounds **16** and **17**, which prevented isolation of a reasonably pure prodrug. To avoid this problem, we performed the reduction step prior to the removal of the 2′,3′-blocking group, as shown in Scheme 3A. Treatment of **11** with sodium borohydride in a two-phase system consisting of pH 7 phosphate buffer and ethyl acetate gave the tetrahydro compound **18**, which could be deblocked with aqueous sulfuric acid to produce prodrug **19**. It is clear that in order to stop the pyridinium salt reduction at the dihydro state in this system, the carbonyl must be conjugated to a double bond in the dihydropyridine system. The prodrug **19** is extremely susceptible to hydrolysis, and our product contains a significant quantity of ribavirin as the major impurity. The other point about **19** and similar compounds is that they cannot participate in the redox reaction that is suggested to be their mechanism of action. Such a prodrug would be a good test of whether that mechanism is truly operative in this situation.

We also attempted to prepare compound **24** by the sequence of reactions shown in Scheme 4. As was the case with the lower homolog **11**, reduction of **23** was difficult to control, and hydrolysis of any of the various esters was facile. In an effort to maintain conjugation of the ester carbonyl with a double bond in the dihydropyridine moiety, we also approached potential prodrug **28** by the route shown in Scheme 5. Attempted reduction of **27** and **28** was not successful because the double bond adjacent to the ester carbonyl reduced prior to the completion of the desired reduction of the pyridinium ring. No further effort was expended on either of the above-mentioned prodrugs.

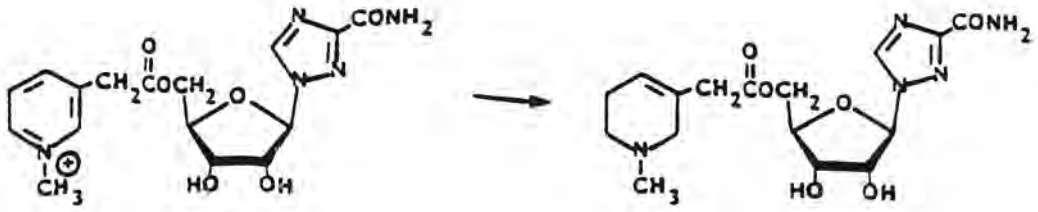
Scheme 2



Scheme 3



Scheme 3A



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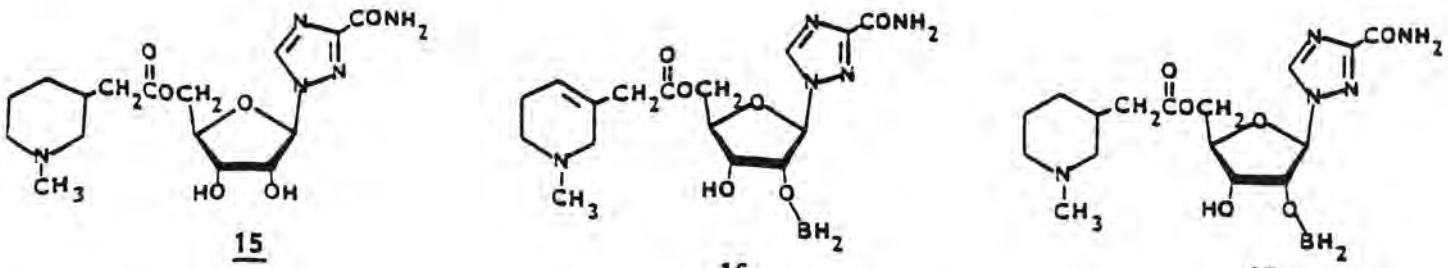
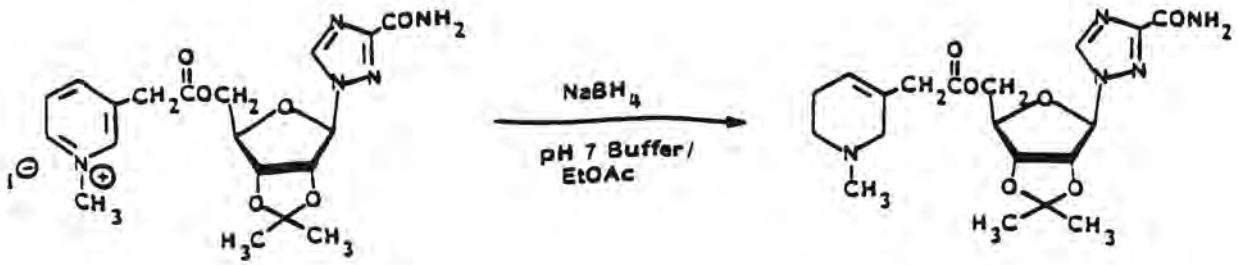
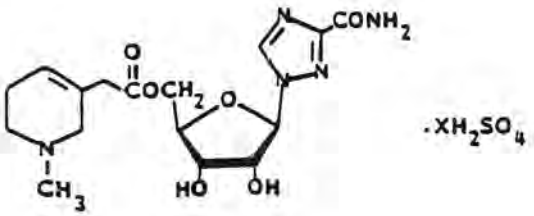


15 16 17



11 18



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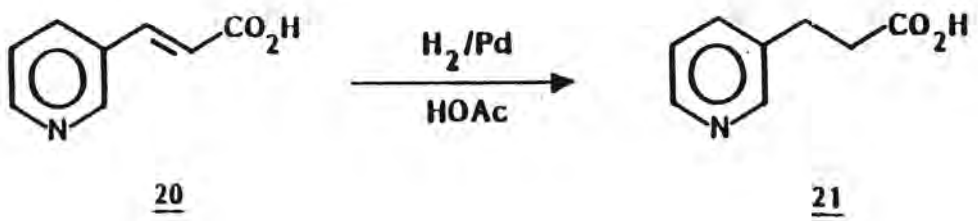
Scheme 4



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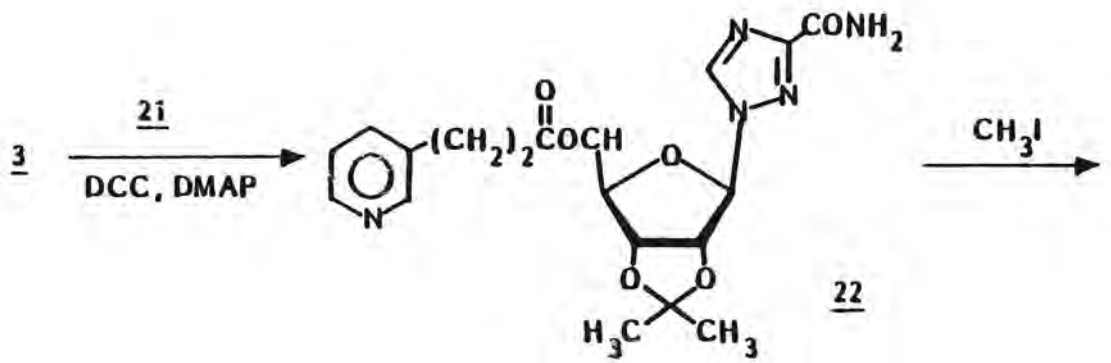

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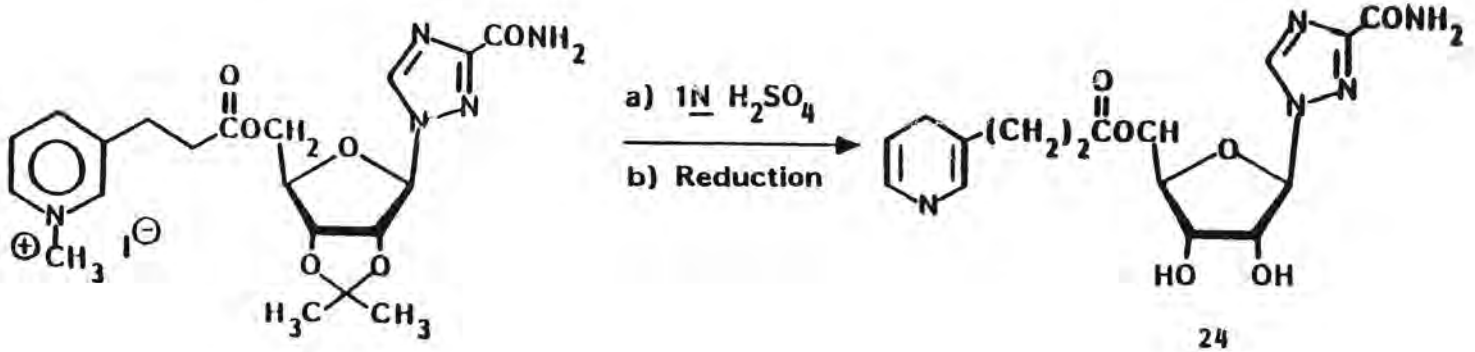
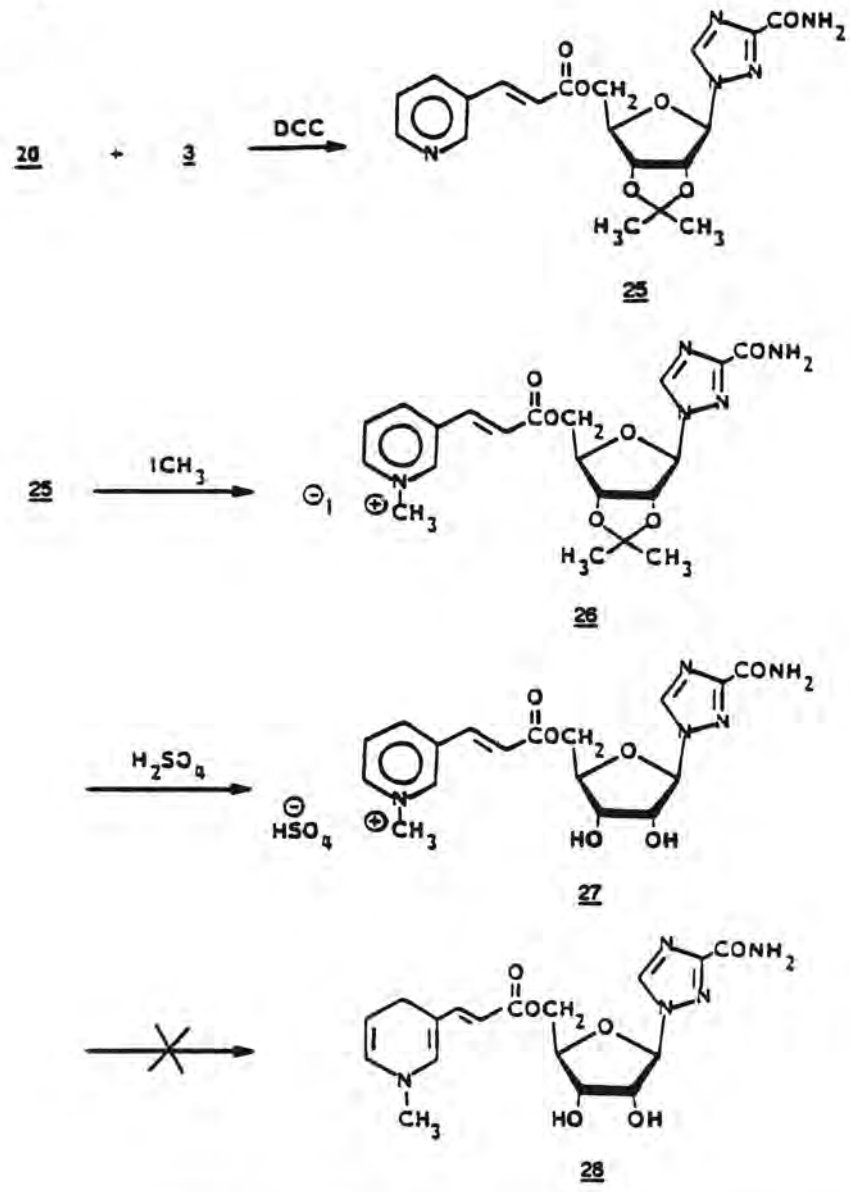
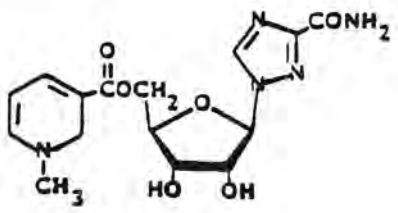


23 24

Scheme 5





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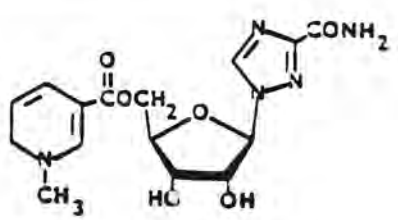
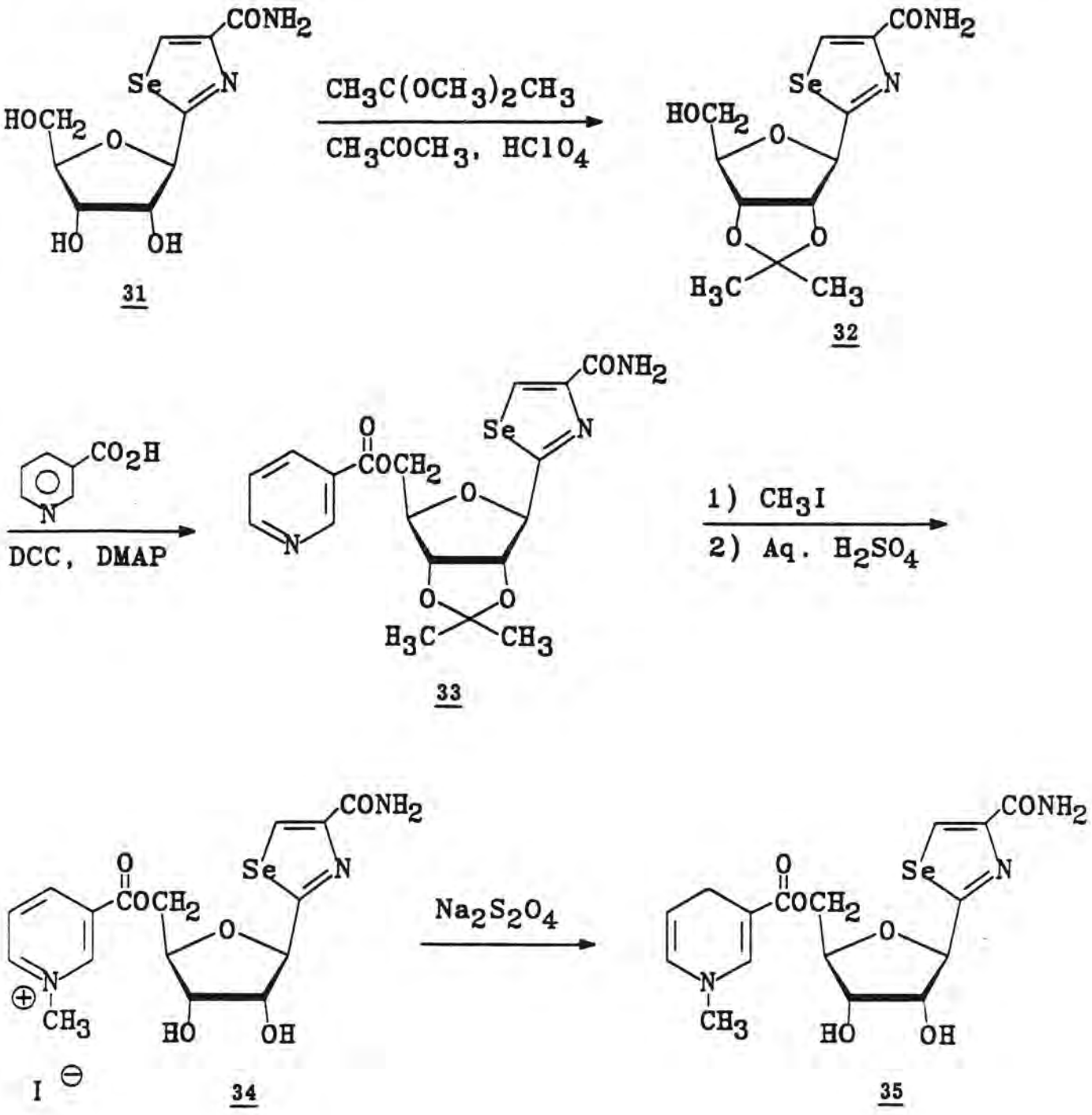
(^{14}C-labelled)



30

(^{14}C-labelled)

Scheme 6


CONH2
Se
N
HOCH2
O
HO
OH
31
CH3C(OCH3)2CH3
CH3COCH3, HClO4
32
CO2H
DCC, DMAP
COCH2
33
1) CH3I
2) Aq. H2SO4
CH3
I ⊖
34
Na2S2O4
35

The synthesis of ^{14}C-labelled **1** was accomplished through the same intermediates shown in Scheme 1, starting with ^{14}C-ribavirin. The sequence was run through first with cold ribavirin on the microscale that would be used for the labelled material, and then the labelled material was made. Significant changes were necessary in the experimental details in several instances in order to successfully obtain labelled **1**, and these changes are noted in the Experimental Section as compounds **3a**, **4a**, **5a**, **6a**, and **1a**. The synthesis provided *ca.* 9 mg of labelled **1a** with a specific activity of 13.6 μCi/mg. The prodrug contained small amounts of ^{14}C-ribavirin as well as labelled **5** and a small amount of both dihydro isomers of **1**, and the 1,2- and 1,6-dihydro isomers **29** and **30**. The existence of the other isomers was only seen by HPLC analysis of the labelled prodrug. They are probably present also in the unlabelled prodrug.

Figures 1-4 provide information on the character and stability of the ^{14}C-labelled prodrug **1a**. Figure 1 shows that the prodrug is actually three isomers, the main one being the expected 1,4-dihydropyridine structure **1a**, and the others presumably the 1,2- and 1,6-dihydropyridines **29** and **30**. The prodrug is quite stable in aqueous solution. Figure 2 depicts the distribution of radiolabelled material showing the isomers (the two major and one minor isomers) and the minor amounts of ribavirin and oxidized prodrug present. Figure 3 shows that oxygen bubbled through an aqueous solution of the prodrug **1a** has little effect. We have found earlier, however, that the prodrug has less stability in a phosphate buffer than it does in pure water. Figure 4 shows the ultraviolet absorption spectra of the three prodrug isomers recorded during their separation by HPLC. Data on the animal distribution studies with this prodrug are given later in the report.

Starting from selenazofurin (**31**), we have prepared the standard prodrug **35** by the route shown in Scheme 6. It is a sensitive compound, hydrolyzing readily back to selenazofurin. Silica gel chromatography, even thin-layer chromatography, results in hydrolysis, so that it is difficult to monitor the reaction by standard means. Analysis by reverse-phase HPLC appears to be the best method.

Experimental Section

1-[5-*O*-(*N*-methyl-1,4-dihydropyridinylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide Monohydrate (1 · H_2O). A solution of **6** (613 mg, 1.33 mmol) and sodium bicarbonate (672 mg, 8 mmol) in 10 mL of deoxygenated H_2O was cooled in an ice bath under a nitrogen atmosphere. Sodium dithionite (820 mg of Aldrich 85% technical grade; 4.0 mmol) was added; after 1 min a precipitate began forming. After 5 min the solid was collected under N_2 and washed thoroughly with deoxygenated H_2O, then quickly transferred and dried under high vacuum at room temperature. The filtrate was applied to a Bio-Bead SM-4 column (1 x 10 cm) and washed with 20 mL H_2O. The Bio-Bead column we prepared by washing first with degassed H_2O, then degassed CH_3OH, then back flushing with degassed water to remove air bubbles, and then thoroughly washing with H_2O. Methanol (50 mL) was then used to elute the product. The solution was evaporated nearly to dryness *in vacuo*; addition of H_2O caused a precipitate which was treated as before. The first crop weighed 350 mg; the second 48 mg (total yield 1.04 mmol, 78%). Both crops were identical by melting point and NMR. Mp 111-113 °C (dec). FABMS: *m/z* 366 $(M + 1)^+$. ^{1}H NMR (Me_2SO-d_6) δ 8.81 (s, 1, H-5), 7.81 and 7.63 (2 s, 2, $CONH_2$), 7.00 (d, 1, H-2'', $J_{2'',4''}$ = 1.5 Hz), 5.88 (d, 1, H-1'), 5.82

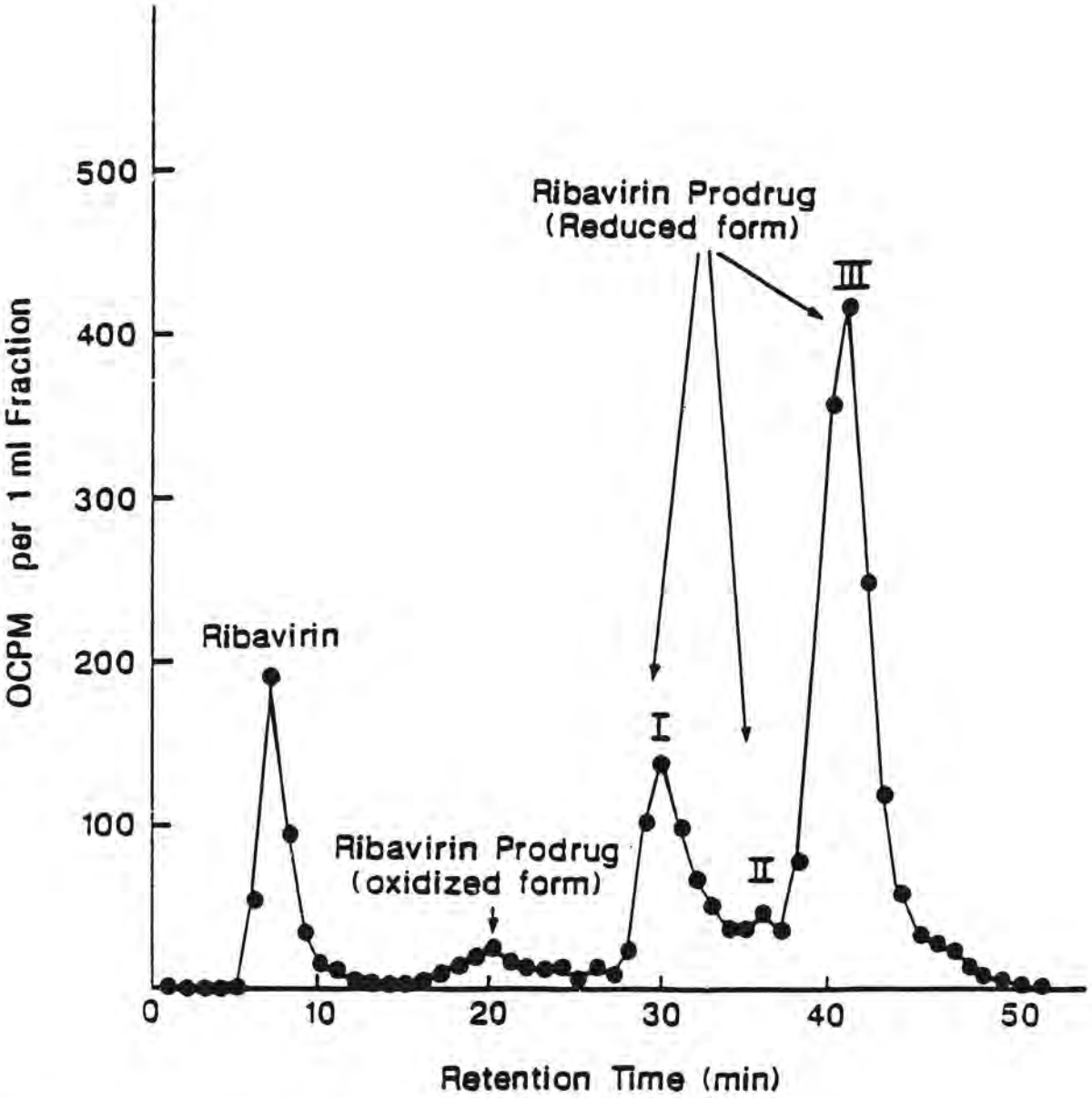


Figure 1. Analysis of [^{14}C] SRI 6711 by HPLC.
[^{14}C] SRI 6711 in H_2O was chromatographed as described in the text. One minute (1 ml) fractions were collected for radioassay. Radioassay data indicated that three radioactive compounds were present: ribavirin (16%) at approximately 8 min and two forms of the reduced prodrug (84%) at approximately 30 and 40 min. Trace amounts of the oxidized prodrug (20 min) and a third form of the reduced prodrug (35 min) were also present.

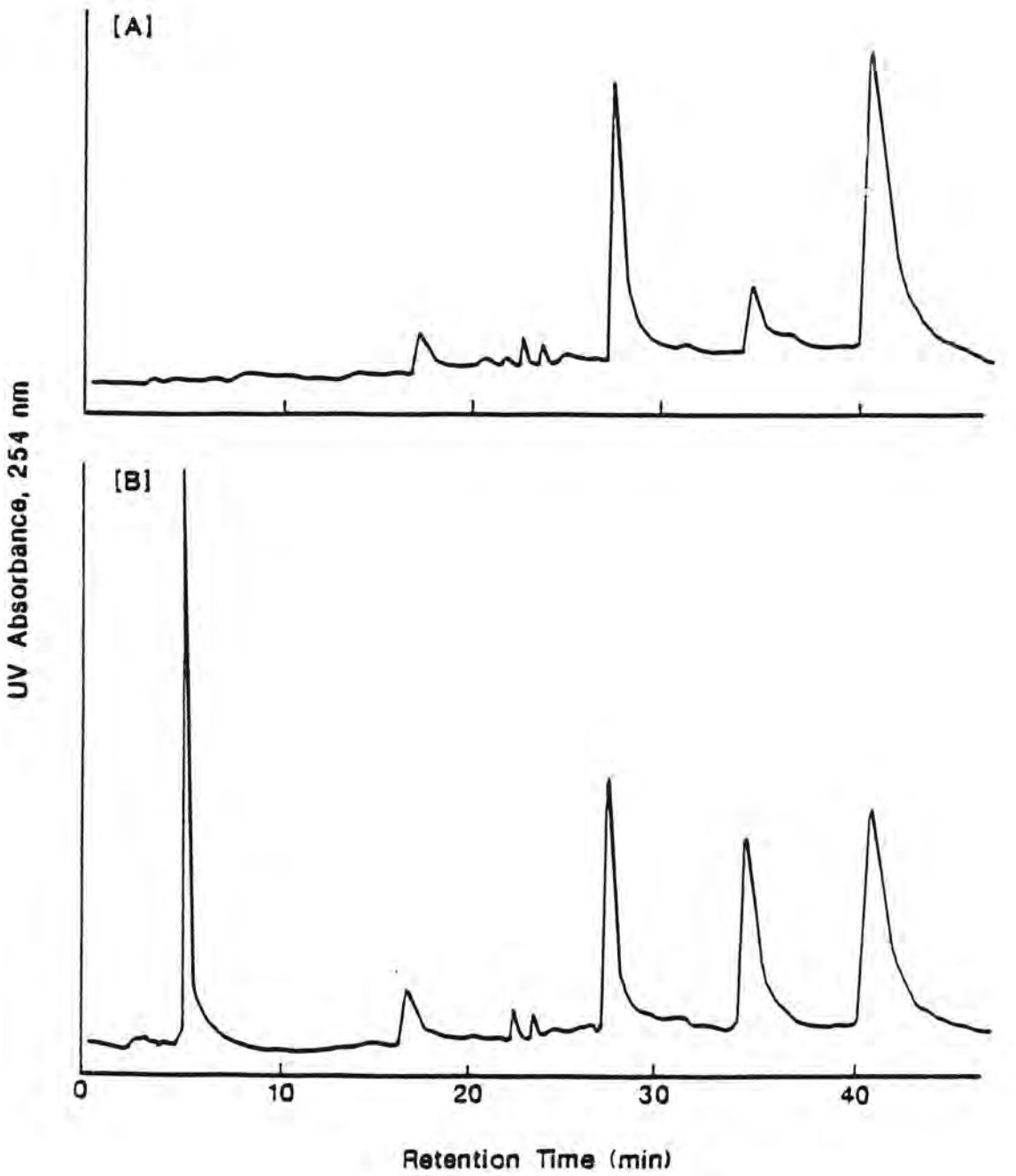


Figure 2. Stability of SRI 6711 in H_2O.
A freshly prepared aqueous solution of SRI 6711 was analyzed by HPLC, stored at -20° C for one week, and reanalyzed. While some breakdown to ribavirin did occur, the most significant change appeared to be the interconversion of the three forms of the reduced prodrug. Under these conditions there was no buildup of the oxidized form of the prodrug. A, HPLC analysis of freshly prepared aqueous solution; B, HPLC analysis of solution after one week at -20° C.

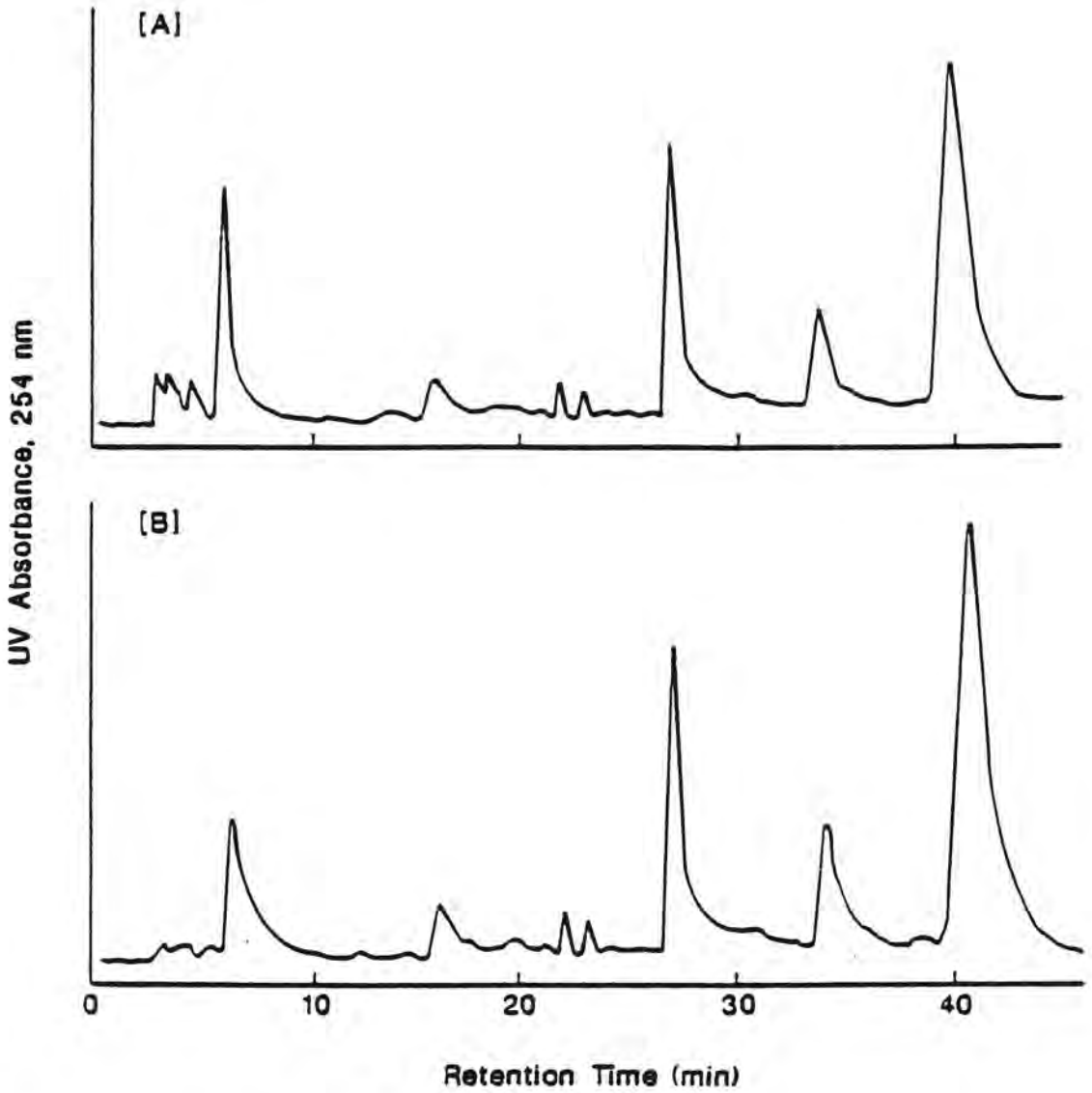


Figure 3. Effect of O_2 on an aqueous solution of SRI 6711. A solution of SRI 6711 in water was treated with O_2 for 15 min. HPLC analyses of the solution before and after this treatment indicated that there were no significant changes in the composition of the sample as a result of this treatment. A, before O_2; B, after O_2.

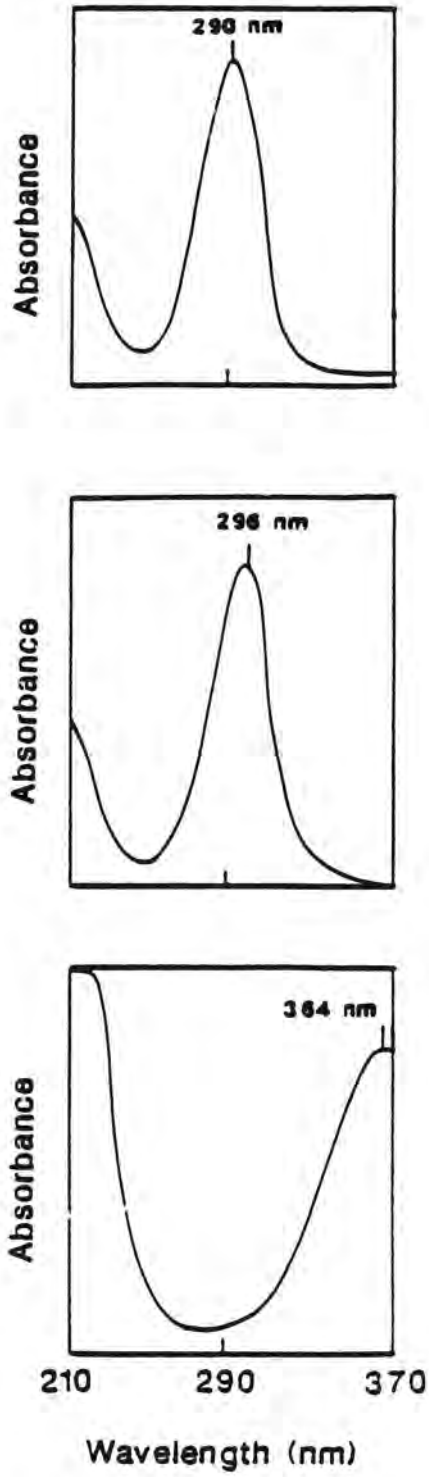


Figure 4. Ultraviolet absorption spectra of the peaks appearing between 25 and 40 min in the chromatograms shown in Figure 2B. The peaks eluting at 27, 34, and 40 min were scanned using an LKB 2140 scanning detector. A, peak eluting at 27 min with a UV max at 290 nm; B, peak eluting at 33 min with a UV max at 296 nm; C, peak eluting at 40 min with UV max at 364 nm.

(complex m, 1, H-6'', $J_{1'',6''}$ = 1.5 Hz, $J_{4'',6''}$ = 1.6 Hz, $J_{5'',6''}$ = 8.0 Hz), 5.64 (d, 1, 2'-OH), 5.35 (d, 1, 3'-OH), 4.71 (dt, 1, H-5'', $J_{4',5''}$ = 3.4 Hz), 4.13 (m, 2, H-5'), 2.94 (s, 3, N-CH_3), 2.93 (m, 2, H-4'', seen as shoulder of N-CH_3 peak). ^{13}C NMR (Me_2SO-d_6) δ 166.65 ($CONH_2$), 160.19 (C-5''), 157.43 (CO_2), 145.27 (C-5), 142.85 (C-2''), 129.76 (C-6''), 103.60 (C-5''), 94.38 (C-3''), 91.26 (C-1'), 81.94 (C-4'), 74.04 (C-3'), 70.27 (C-2'), 62.84 (C-5'), 40.05 (N-CH_3), 21.52 (C-4''). *Anal.* Calcd for $C_{15}H_{19}N_5O_6 \cdot H_2O$: C, 47.00; H, 5.52; N, 18.27. Found: 46.90; H, 5.55; N, 18.18. UV (H_2O) λ_{max} 367 (7500).

1-[5-*O*-(*N*-Methyl-1,4-dihydropyridinylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide-^{14}C (1a). A solution of **6a** (0.051 mmol) in 3 mL of deoxygenated H_2O was cooled in an ice bath under an argon atmosphere. To the cold solution was added sodium bicarbonate (25 mg, 0.30 mmol) followed quickly by 54 mg (0.31 mmol) of sodium dithionite. The reaction solution was kept in the cold for 5 min before applying to a Bio-Bead SM-4 column (0.5 x 6 cm, 1.4 g). [The column was prepared by washing with degassed H_2O (60 mL), then degassed MeOH (60 mL), then back flushing with degassed H_2O to remove air bubbles, followed by washing well with degassed H_2O (100 mL).] The column was first eluted with degassed H_2O (50 mL). Degassed MeOH (25 mL) was used to elute the product, which was collected under argon and quickly evaporated to dryness *in vacuo*. A yellow residue was obtained, 9.6 mg (50.5%). It was redissolved in 1 mL of degassed MeOH for assay. Three 1-μL fractions were removed for scintillation counting and 1 mL for TLC. The remainder of the solution was evaporated to dryness *in vacuo* and stored at -80 °C under argon. The material was found by TLC (3:1 $CHCl_3$-MeOH + 5% 2-mercaptoethanol) to contain by UV examination **5** plus starting compound. Radioactivity scans of the thin-layer chromatogram with a Packard radiochromatogram scanner, Model 7201 showed ribavirin (0.5%), starting or oxidized compound (10.8%), and prodrug **5** (88.7%). The specific activity determined by liquid scintillation counting was 13.6 μCi/mg or 4.98 mCi/mmol. FABMS: *m/z* 364 (cation), 366 $(M + 1)^+$. HPLC with a different solvent system showed that a second isomer of the dehydropyridine was also present in the prodrug.

1-(2,3-*O*-Methylethylidene-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide (3). A suspension of ribavirin (3.00 g, 12.3 mmol) in 2,2-dimethoxypropane (20 mL) and dry acetone (40 mL) was cooled to 0 °C, and 70% perchloric acid (0.6 mL) was then added. The mixture was allowed to warm to room temperature while stirring for 3 h. TLC ($CHCl_3$-MeOH, 9:1) showed that all of the starting material was gone, with the production of one major product plus a faster-traveling minor by-product. The solution was neutralized with 1*N* NaOH and evaporated to dryness *in vacuo*. The residue was triturated with methanol (20 mL) and filtered. The filtrate was concentrated and the product crystallized from CH_3OH-EtOAc. The faster-traveling impurity was still present, so the mixture was purified by flash chromatography (EtOAc-CH_3OH, 9:1). Precipitation from methanol with ethyl acetate gave 2.98 g (10.5 mmol, 85%), homogeneous by TLC, mp 152-154 °C (lit. mp 153-154 °C). The compound was used directly for further reactions.

1-β-(2,3-*O*-Methylethylidene-β-D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide-^{14}C (3a). A solution of 3.3 mg (0.013 mmol) of ^{14}C-ribavirin (1) (448 μCi, SA 33 mCi/mmol, ICN Lot #2414134) in 40% ethanol (5 mL, v/v) was diluted with 9.9 mg (0.041 mmol) of ribavirin (total, 0.054 mmol). The resulting solution was

evaporated to dryness *in vacuo*. The residue was triturated with anhydrous ethanol and the mixture evaporated to dryness three times. The residue was dried for 20 h at ambient temperature and 0.07 mm before it was suspended in 4.5 mL of anhydrous acetone containing 89 mL of 2,2-dimethoxypropane followed by 2.7 mL of 70% perchloric acid. A complete solution was obtained after about one hour at ambient temperature. After 18 h, the solution was made basic with an excess of conc. NH_4OH and evaporated to dryness *in vacuo*. The residue was triturated with anhydrous ethanol, the mixture evaporated to dryness two times, and the residue was dried for 2 h at ambient temperature and 0.07 mm. It was homogeneous by TLC ($CHCl_3$-MeOH, 9:1, R_f 0.36) and of greater than 97% radiochemical purity, determined by radioactivity scans of the thin-layer chromatogram with a Packard radiochromatogram scanner, Model 7201.

The residue was used in the next step without further purification.

1-[2,3-*O*-Methylethylidene-5-*O*-(3-pyridinylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide (4). A mixture of 2′,3′-isopropylideneribavirin (**2**, 1.00 g, 3.52 mmol, ref. 1), nicotinic acid (435 mg, 3.54 mmol), and 4-dimethylaminopyridine (43 mg, 0.35 mmol) were stirred in THF (25 mL) at room temperature. Dicyclohexylcarbodiimide (740 mg, 3.6 mmol) was then added in one portion, and stirring continued for 1 h. The suspended solid was filtered off and washed thoroughly with ethyl acetate. The combined filtrate and washings were concentrated *in vacuo* and purified by flash chromatography (EtOAc-EtOH, 9:5). The collected fractions gave a white foam, homogeneous by TLC, 1.09 g (2.80 mmol, 80%). An analytical sample was crystallized from a small amount of water, mp 92-95 °C (dec). FABMS: *m/z* 390 $(M + 1)^+$. ^1H NMR (Me_2SO-d_6) δ 9.00 (d, 1, 2″-C*H*), 8.81 (m, 1, 5-C*H*), 8.79 (d, 1, H-6″), 8.20 (dt, 1, H-4″), 7.81 and 7.64 (2 s, 2, $CONH_2$), 7.54 (m, 1, H-5″), 6.34 (dt, 1, H-1′), 5.29 (d, 1, H-2′), 5.17 (dd, 1, H-3′), 4.64 (m, 1, H-4′), 4.53-4.38 (m. 2, 5′-CH_2), 1.52 and 1.34 (2 s, 6, -C(CH_3)$_2$). *Anal.* Calcd for $C_{17}H_{19}N_5O_6 \cdot H_2O$: C, 50.12; H, 5.19; N, 17.19. Found: C, 49.83; H, 5.00; N, 17.54.

1-[2,3-*O*-Methylethylidene-5-*O*-(3-pyridinylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide-^{14}C (4a). A solution of **3a** (0.054 mmol), nicotinic acid (13.3 mg, 0.108 mmol) and dicyclohexylcarbodiimide (89 mg, 0.432 mmol) in THF (3.6 mL, Aldrich Gold Label) was treated with 4-dimethylaminopyridine (6.6 mg, 0.054 mmol) and stirred 20 h at ambient temperature. The resulting cloudy solution was evaporated to dryness *in vacuo*. A solution of the residue in 1 mL of $CHCl_3$-MeOH (1:1) was streaked across one Brinkmann Silica Gel 60 F^{254} 2 mm plate (20 x 20 cm). The plate was developed in $CHCl_3$-MeOH (9:1). The product band was extracted with $CHCl_3$-MeOH (2:1) (about 30 mL). Evaporation of the extract gave a glass, 20 mg. It was homogeneous by TLC (9:1 $CHCl_3$-MeOH, R_f 0.44) and of greater than 99% radiochemical purity, determined by radioactivity scans of the thin-layer chromatogram with a Packard radiochromatogram scanner, Model 7201.

1-[2,3-*O*-Methylethylidene-5-*O*-(*N*-methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide Iodide (5). A solution of **4** (100 mg, 0.26 mmol) and methyl iodide (65 μL, 1.05 mmol) in acetone (10 mL) was refluxed (70 °C oil bath) for 20 h, employing an efficient condenser. The solution was cooled, and the product precipitated with ether and collected. Recrystallization from acetone-ether gave 105 mg (0.198 mmol), 76%) as a yellow solid. FABMS: *m/z* 404 (M^+ of cation). ^1H NMR (Me_2SO-d_6) δ 9.48

(s, 1, H-2″), 9.18 (d, 1, H-6″), 8.95 (d, 1, H-4″), 8.83 (s, 1, H-5), 8.24 (m, 1, H-4″), 7.84 and 7.62 (2 s, 2, $CONH_2$), 6.33 (d, 1, H-1′), 5.31 (dd, 1, H-2′), 5.21 (dd, 1, H-3′), 4.66 (m, 2, H-4′ and H-5a′), 4.52 (dd, 1, H-5b′), 4.44 (s, 3, NCH_3), 1.56 and 1.37 (2 s, 6, $C(CH_3)_2$).

1-[2,3-*O*-Methylethylidene-5-*O*-(*N*-methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide-^{14}C Iodide (5a). A solution of **4a** (20 mg, 0.051 mmol) in acetone (5 mL, anhydrous) containing methyl iodide (51 mg, 0.36 mmol) was heated in a tightly sealed flask at 40 °C for 20 h. Examination of the reaction mixture by TLC (BuOH-HOAc-H_2O, 5:2:3) showed about 40% product formed. Another 51 mg of methyl iodide was added followed by diisopropylethylamine (4 mg, 0.031 mmol) and heating was resumed for 20 h. TLC examination showed about 75% product formed. Therefore, another 51 mg of methyl iodide was added followed by diisopropylethylamine (1.3 mg, 0.01 mmol) and heating resumed for 20 h. Again TLC showed starting compound (10%). Another 51 mg of methyl iodide was added followed by diisopropylethylamine (0.7 mg, 0.005 mmol) and heating resumed for 20 h. The solution was evaporated to dryness, giving a glass that was homogeneous by TLC and of greater than 99% radiochemical purity.

It was used in a reaction without further purification.

1-[5-*O*-(*N*-Methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide Sulfate Salt (6). A solution of **5** (2.25 g, 4.23 mmol) in 1*N* H_2SO_4 (20 mL) was stirred at room temperature for 18 h. The volume was reduced to one half on a rotary evaporator, and 200 mL of EtOH was added. Storage in the freezer for two days yielded a pale yellow solid, 1.88 g (4.08 mmol; 96%), mp 177-179 °C (dec). FABMS: *m/z* 364 (M^+). ^{1}H NMR (Me_2SO-d_6) δ 9.50 (s, 1, H-2″), 9.21 (d, 1, H-6″), 9.05 (d, 1, H-4″), 8.88 (s, 1, H-5), 8.26 (dd, 1, H-5′), 7.88 and 7.64 (2 s, 2, $CONH_2$), 5.98 (d, 1, H-1′), 4.76-4.48 (m, 3, H-3′ and H-5′), 4.46 (s, 3, N-CH_3), 4.42 (m, 1, H-2′), 4.32 (m, 1, H-4′). *Anal.* Calcd for $C_{15}H_{18}N_5O_6^+ \cdot HSO_4^-$: C, 39.05; H, 4.15, N, 15.18; S, 6.95. Found: C, 38.90; H, 4.18; N, 15.02; S, 6.88.

1-[5-*O*-(*N*-Methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide-^{14}C Sulfate Salt (6a). A solution of 20 mg (0.051 mmol) of **5a** in 1*N* H_2SO_4 (0.36 mL) was kept 20 h at ambient temperature, decanted from some orange waxy insolubles, diluted with 3 mL of H_2O and neutralized with 71 mg (0.36 mmol) of $BaCO_3$. The precipitate of $BaSO_4$ was removed by filtration and washed well with H_2O. The combined filtrate and wash was evaporated to dryness *in vacuo*. A glass was obtained that was found by TLC (BuOH-HOAc-H_2O, 5:2:3) and radioactivity scans of the thin-layer chromatogram to contain starting compound (about 2%), ribavirin (10%), and **5** (88%).

5′-O-(3-Pyridinylcarbonyl)ribavirin (8). To a solution of nicotinic acid (**7**, 93 mg, 0.5 mmol), ribavirin (**2**, 122 mg, 0.5 mmol) and triphenylphosphine (197 mg, 0.75 mmol) in 5 mL of DMF was added DEAD (118 mL, 0.75 mmol). After 4 h an additional equivalent each of triphenylphosphine and DEAD was added. The solution was then stirred at room temperature overnight. The solvent was evaporated (1 torr; room temperature) and the residue dissolved in a minimal amount of hot ethanol, from which the product crystallized. The product was collected and washed with EtOH. The filtrate was concentrated and a second crop was obtained identical to the first to give a total of 133 mg (0.32 mol; 64%) homogeneous by TLC ($CHCl_3$-MeOH, 3:1). Mp 180-182 °C. FABMS: 350 $(M + 1)^+$. ^{1}H NMR (Me_2SO-d_6) δ 9.08 (s, 1, H-

2''), 8.88 (s, 1, H-5), 8.78 (d, 1, H-6''), 8.35 (d, 1, H-4''), 7.85 and 7.68 (2 s, 2, -C=O-NH_2), 7.55 (m, 1, H-5''), 5.95 (d, 1, H-1'), 5.74 (d, 1, HO-2'), 5.45 (d, 1, HO-3'), 4.60-4.35 (m, 4, H-2', H-3', and H-5'), 4.26 (s, 1, H-4'). HPLC (254 nm) 99%.

The reaction was repeated on a 20.5 mmol scale to yield 3.31 g (8.1 mmol; 40%) of material with similar analytical data.

5'-O-(1-Methyl-3-pyridiniumcarbonyl)ribavirin Iodide (9). A solution of **8** (1.11 g, 2.70 mmol) and iodomethane (0.4 mL, 3.2 mmol) in 15 mL of DMAC was stirred for 16 h, at which time 0.15 mL more ICH_3 was added and stirring continued at room temperature for 24 h. The product was precipitated with ether, collected and rinsed with ether to give 1.46 g (2.64 mmol; 93%) of a hygroscopic yellow solid. FABMS 364 M^+ of cation. ^1H NMR (Me_2SO-d_6) δ 9.50 (s, 1, H-2''), 9.22 (d, 1, H-6''), 9.05 (d, 1, H-4''), 8.88 (s, 1, H-5), 8.28 (m, 1, H-5''), 7.90 and 7.65 (2 s, 2, -C=O-NH_2), 5.95 (d, 1, H-1'), 5.72 (d, 1, HO-2'), 5.45 (d, 1, HO-3'), 4.72 (dd, 1, H-5a'), 4.55 (m, 2, H-5b' and H-3'), 4.48 (m, 1, H-2'), 4.46 (s, 3, NH^+-CH_3), 4.32 (m, 1, H-4').

1-[2,3-*O*-Methylethylidene-5-*O*-[3-(pyridylmethyl)carbonyl]-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide (10). A mixture of **3** (2.97 g, 10.45 mmol), (3-pyridyl) acetic acid hydrochloride (2.00 g, 11.50 mmol), and 4-dimethylaminopyridine (0.43 g, 3.50 mmol) was stirred in dry THF (100 mL) at room temperature. Dicyclohexylcarbodiimide (4.30 g, 20.88 mmol) was added and stirring continued for two days. The suspended solid was filtered and washed with ethyl acetate. The combined filtrates were concentrated under reduced pressure and purified by flash chromatography ($CHCl_3$-MeOH, 95:5). The homogeneous fractions were combined and dried (Na_2SO_4) to give 2.76 g (65%) of a white foam, which was taken up in $CHCl_3$ and precipitated with petroleum ether. This tacky material was suitable for use in the next step. FABMS: *m/z* 404 $(M + 1)^+$. ^1H NMR ($CDCl_3$) δ 8.53 (dd, 1, H-6''), 8.51 (d, 1, H-2''), 8.31 (s, 1, H-5), 7.62 (dt, 1, H-4''), 7.29 (dd, 1, H-5''), 7.25 and 6.46 (2 s, 2, $CONH_2$), 6.08 (d, 1, H-1'), 5.33 (dd, 1, H-2'), 4.89 (dd, 1, H-3'), 4.55 (m, 1, H-4'), 4.29 (dd, 1, H-5a'), 4.21 (dd, 1, H-5b'), 3.63 (s, 2, -CH_2C=O-O), 2.27 (impurity ? exchanges with D_2O), 1.58 and 1.36 (2 s, 6, $C(CH_3)_2$).

1-[2,3-*O*-Methylethylidene-5-*O*-[N-methyl[(3-pyridiniumylmethyl)carbonyl]]-β-D-ribofuranosyl]-1*H*-1,2,4-triazole-3-carboxamide Iodide (11). A solution of **10** (2.49 g, 6.17 mmol) and iodomethane (1.43 mL, 22.7 mmol) in 90 mL of acetone was refluxed in a 70 °C oil bath. After 5 h an orange-yellow precipitate (gummy residue) had formed, from which the yellow solution was decanted. This solution was concentrated *in vacuo* to 3/4 of the original volume and the product was precipitated with 200 mL of ether. The product was collected by filtration, rinsed with ether and dried *in vacuo* to give a light, yellow powder, 1.43 g. The orange-yellow residue was collected as an orange foam, 1.32 g, which was identical to the light yellow precipitate by mass spectral analysis. Total yield, 2.75 g (82%). FABMS: *m/z* 418 (M^+ of cation). ^1H NMR (Me_2SO-d_6) δ 8.96 (s, 1, H-2''), 8.93 (d, 1, H-6''), 8.86 (s, 1, H-5), 8.50 (d, 1, H-4''), 8.12 (dd, 1, H-5''), 7.94 and 7.72 (2 s, 2, $CONH_2$), 6.35 (d, 1, H-1'), 5.21 (dd, 1, H-2'), 5.04 (dd, 1, H-3'), 4.48 (m, 1, H-5a'), 4.35 (s, 3, N+CH_3), 4.31 (d, 1, H-4'), 4.17 (dd, 1, H-5b'), 4.04 (s, 2, -CH_2C=O-O-), 1.52 and

1.33 (2 s, 6, $C(CH_3)_2$). *Anal.* Calcd for $C_{19}H_{24}IN_5O_6$: C, 41.84; H, 4.44; N, 12.84. Found: C, 41.44; H, 4.57; N, 12.43.

1-[5-O-[N-methyl-[(3-pyridiniumylmethyl)carbonyl]]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Sulfate (12). Compound **11** (1.72 g, 3.16 mmol) was stirred in 15 mL of 1*N* H_2SO_4 for 16 h at room temperature. The solution was concentrated to one-half volume and 80 mL of anhydrous EtOH was added. The resulting suspension was placed in the freezer for 1 week to give an off-white film. The solution was decanted and the film dissolved in 10 mL of H_2O and precipitated again with EtOH to give 432 mg (0.91 mmol, 29%) of an off-white powder. FABMS: *m/z* 378 (M^+ of cation). ^1H NMR (Me_2SO-d_6) δ 8.99 (s, 1, H-2''), 8.92 (d, 1, H-6''), 8.86 (s, 1, H-5), 8.50 (d, 1, H-4''), 8.10 (t, 1, H-5''), 7.94 and 7.70 (2 s, 2, $CONH_2$), 5.91 (d, 1, H-1'), 4.9-4.6 (s, +H, -OH's, NH's), 4.45 (dd, 1, H-2'), 4.35 (s, 3 N-CH_3), 4.30 (m, 1, H-3'), 4.13 (t, 1, H-4'), 4.10 (m, 2, H-5a' + 5b'), 4.05 (s, 2, -CH_2COO-). HPLC 95%; mp 97-101 °C dec.

1-[5-O-(1,2,5,6-Tetrahydro-1-methyl-3-pyridinylacetyl)-2,3-O-(methylethylidene)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (18). Compound **18** (360 mg, 0.661 mmol) was dissolved in 5 mL of phosphate buffer (pH 7). Ethyl acetate was added and the two-phase reaction medium was stirred at room temperature. Sodium borohydride (150 mg, 3.95 mmol) was added in one portion under argon and stirring was continued for 25 min. The organic layer was removed and washed with saturated sodium chloride. This aqueous layer was then back-extracted with ethyl acetate. The ethyl acetate was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by preparative thin-layer chromatography using chloroform/methanol (3:1) as eluant. ^1H NMR (300 MHz, $CDCl_3$) δ 1.48, 1.58 (s, 6, OCH_3), 2.20 (bs, 2, H-5 of 6-membered heterocycle), 2.34 (s, 3, N-CH_3 of 6-membered heterocycle), 2.48 (t, 2, H-6 of 6-membered heterocycle), 2.85, 2.92 (d, 4, H-2 and 3-CH_2 of 6-membered heterocycle), 4.2 (m, 2, 4'-CH_2), 4.55 (h, 1, 4'-H), 4.92 (dd, 1, H-3'), 5.38 (dd, 1, H-2'), 5.58 (bs, 1, H-4 of 6-membered heterocycle), 5.58 (s, 1, H-1'), 6.38, 7.16 (bs, 2, $CONH_2$), 8.33 (s, 1, CH of triazole).

1-[5-O-(1,2,5,6-Tetrahydro-1-methyl-3-pyridinylacetyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (19). Compound **18** (1.05 g, 0.002 mol) was stirred in 20 mL of 1*N* H_2SO_4 for 6 h. The reaction was concentrated to about 14 mL and then acetonitrile was added to precipitate the compound as an oil. The residue was lyophilized to provide 441 mg of a glass-like solid which proved to be a 60:40 mixture of **19** and (**2**) ribavirin. ^1H NMR (300 MHz, Me_2SO-d_6) δ 2.34-4.36 (multiplets, 6-membered heterocycle protons and N-CH_3), 3.4-4.5 (multiplets, sugar protons), 5.79 (multiplet, 2'-H and HC=C of the 6-membered heterocycle), 6.5 (vb s, NH, OH, H_2), 6.95, 7.13, 7.31 (s, NH^+), 7.77 (bs, $CONH_2$), 8.84 (s, C-H of 5-membered heterocycle).

3-(3-Pyridyl)propanoic Acid (21). 3-(3-Pyridyl)acrylic acid (**20**, 2.50 g, 16.76 mmol) and 35 mg of 10% Pd on carbon were stirred at room temperature, under 1 atm H_2 in 4 mL of glacial acetic acid for 20 min. The catalyst was filtered and the filtrate was evaporated (20 mm Hg, 50 °C) to give a crystalline solid. The solid was recrystallized from hot EtOH to give 2.10 g (13.89 mmol, 83%) of a white crystalline solid. Mp 152- 153 °C (lit. 160-162 °C) (15); EIMS 151 (M), 133 $(M - H_2O)^+$; ^1H NMR (Me_2SO-d_6) δ 12.2 (s, 1,

-OH), 8.45 (s, 1, H-2), 7.65 (d, 1, H-4), 7.30 (dd, 1, H-5), 2.85 (t, 2, -$CH_2$$CH_2$COOH), 2.58 (t, 2, -$CH_2CH_2$COOH). *Anal.* Calcd for $C_8H_9NO_2 \cdot 0.03EtOH \cdot 0.03CH_3CO_2H$: C, 63.18; H, 6.08; N, 9.08. Found: C, 63.31; H, 6.23; N, 8.90.

1-[2,3-O-Methylethylidene-5-O-[3-(pyridylethyl)carbonyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (22). 2',3'-*O*-Methylethylideneribavirin (**3**, 100 mg, 0.373 mmol), 3-(3-pyridyl)propanoic acid (57 mg, 0.377 mmol), dicyclohexylcarbodiimide (DCC, 78 mg, 0.377 mmol), and DMAP (46 mg, 0.377 mmol) were stirred in 10 mL anhydrous THF. After 3 h the reaction appeared about 50% complete by TLC ($CHCl_3$-CH_3OH, 9:1). Thus 46 mg more of DMAP and 39 mg of DCC were added. After 1 h no apparent progress was noted. An additional quantity (30 mg) of 3-(3-pyridyl)propanoic acid was then added and the solution was stirred overnight. The urea was filtered off and the solvent was evaporated (20 mm Hg, 35 °C). Purification was accomplished by flash chromatography (30 g silica gel, $CHCl_3$-CH_3OH, 95:5) to give 136 mg (0.339 mmol, 91%) of a white foam, homogeneous by TLC. ^{1}H NMR ($CDCl_3$) δ 8.55 (m, 2, H-2'' and H-6''), 8.28 (s, 1, H-5), 7.52 (d, 1, H-4''), 7.22 (dd, 1, H-5''), 7.13 (s, 2, $CONH_2$), 6.06, (s, 1, H-1'), 5.36 (dd, 1, H-2'), 4.82 (dd, 1, H-3'), 4.52 (m, 1, H-4'), 4.18 (m, 2, H-5a' + 5b'), 2.90 (t, 2, -$CH_2$$CH_2$COO-), 2.58 (t, 2, -$CH_2$$CH_2$COO-), 1.58 and 1.36 (2 s, 6, $C(CH_3)_2$).

1-[5-O-[N-Methyl-[(3-pyridiniumylethyl)carbonyl]]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Iodide (23). To a refluxing solution of **22** (2.00 g, 4.79 mmol) in 200 mL of dry acetone was added iodomethane (1.9 mL, 30.55 mmol). The solution was stirred at reflux in a 70 °C oil bath for 4 h at which time the volume was evaporated to 2/3 the original volume and the product was precipitated with ether to give 2.40 g of a yellow powder homogeneous by TLC (BuOH-HOAc-H_2O, 5:2:3) (4.29 mmol, 90%). ^{1}H NMR (Me_2SO-d_6) δ 8.95 (s, 1, H-2''), 8.85 (d, 1, H-6''), 8.82 (s, 1, H-5), 8.46 (d, 1, H-4''), 8.14 (dd, 1, H-5''), 7.90, 7.67 (2 s, 2, -C=O-NH_2), 6.3 (s, 1, H-1'), 5.21 (d, 1, H-2'), 4.97 (dd, 1, H-3'), 4.42 (m, 1, H-4'), 4.32 (s, 3, N+-CH_3), 4.21 (dd, 1, H-5a'), 4.10 (dd, 1, H-5b'), 3.00 (t, 2, -$CH_2$$CH_2$C=O-O-), 2.75 (m, 2, -$CH_2CH_2$C=O-O-), 1.51, 1.32 (2 s, 6, $C(CH_3)_2$). ^{13}C NMR (Me_2SO-d_6) δ 171.0 (-C=O-O), 160.0 (-C=O-NH_2), 157.4 (C-3), 145.5 (C-5), 145.0 (C-6''), 144.7 (C-2''), 143.1 (C-4''), 140.7 (C-3''), 127.0 (C-5''), 112.7 ($O_2C(CH_3)_2$), 91.9 (C-1'), 85.0 (C-4'), 83.8 (C-2'), 81.1 (C-3'), 63.9 (C-5'), 47.7 (N^+-CH_3), 32.8 (-CH_2CH_2C=O-O-), 26.6 (-CH_2CH_2C=O-O-), 26.6, 24.9 ($C(CH_3)_2$). Mp decomposes from 110 °C. UV λ_{max} 264.5 and 220 nm at pH 1, 264.5 and 220 nm at pH 7, 266 and 222 nm at pH 13. FABMS: 432 (M^+) of cation. *Anal.* Calcd for $C_{20}H_{26}N_5IO_6 \cdot 1H_2O$: C, 41.60; H, 4.90; N, 12.13. Found: C, 41.72; H, 4.92; N, 11.93.

1-[2,3-O-Methylethylidene-5-O-[3-(3-pyridinyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (25). To a mixture of 2',3'-*O*-methylethylideneribavirin (**3**, 1.50 g, 5.28 mmol), 3-(3-pyridyl)acrylic acid (**20**, 1.03 g, 6.91 mmol) and 4-dimethyl aminopyridine (0.843 g, 6.91 mmol) in 200 mL of THF was added DCC (1.56 g, 7.59 mmol). The mixture was stirred for 16 h at room temperature then the resulting DCU was filtered. The filtrate was concentrated and more DCU was filtered off. Then the filtrate was evaporated to dryness *in vacuo* at room temperature and the product was purified by flash chromatography ($CHCl_3$-MeOH, 95:5) and recrystallized from MeOH-$CHCl_3$ to give 1.84 g (4.43 mmol, 84%) of a white crystalline solid. Mp

187 °C; FABMS: 416 $(M + 1)^+$, 304 $(sugar)^+$. ^1H NMR (Me_2SO-d_6) δ 8.87 (d, 1, H-2″), 8.83 (s, 1, H-5), 8.59 (dd, 1, H-6″), 8.26 (d, 1, H-4″), 7.86 and 7.65 [2 s, 2, -C(=O)NH_2], 7.61 [d, 1, -C*H*=CH-C(=O)-O], 7.45 (dd, 1, H-5″), 6.71 [d, 1, -CH=C*H*-C(=O)-O], 6.32 (s, 1, H-1′), 5.29 (d, 1, H-2′), 5.18 (dd, 1, H-3′), 4.54 (m, 1, H-4′), 4.35 and 4.25 [2 dd, 2, -C(=O)-OCH_2], 1.52 and 1.35 (2 s, 6, $C(CH_3)_2$). *Anal.* Calcd for $C_{19}H_{21}N_5O_6$: C, 54.93; H, 5.11; N, 16.86. Found: C, 55.25; H, 5.29; N, 16.98.

1-[2,3-O-Methylethylidene-5-O-[3-(N-methyl-3-pyridiniumyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Iodide (26). Compound **25** (700 mg, 1.69 mmol) and iodomethane (0.53 mL, 8.50 mmol) in 100 mL of acetone were heated at 70 °C in a pressure vessel for 16 h. The solid was collected by filtration and rinsed with acetone and dried under reduced pressure over P_2O_5. A second crop of product was precipitated from the filtrate with ether to give a total yield of 896 mg (1.61 mmol, 95%) of a yellow solid. Mp decomposes from 165 °C; FABMS: 430 $(M)^+$ of cation. ^1H NMR (Me_2SO-d_6) δ 9.42 (s, 1, H-2″), 9.20 (d, 1, H-6′), 8.89 (d, 1, H-4″), 8.85 (s, 1, H-5), 8.19 (dd, 1, H-5″), 7.88 and 7.62 [2 s, 2, -C(=O)-NH_2], 7.70 (d, 1, -C*H*-CHCOO), 6.95 (d, 1, -CH=C*H*COO), 6.35 (s, 1, H-1′), 5.35 (d, 1, H-2′), 5.12 (d, 1, H-3′), 4.59 (m, 1, H-4′), 4.42 and 4.30 (2 dd, 2, H-5′), 4.40 (s, 3, N-CH_3), 1.55 and 1.40 (2 s, 6, $C(CH_3)_2$). *Anal.* Calcd for $C_{20}H_{24}N_5IO_6$: C, 43.09; H, 4.35; N, 12.57. Found: C, 42.40; H, 4.60; N, 12.26.

1-[5-O-[3-(N-methyl-3-pyridiniumyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamideSulfate (27). Compound **26** (577 mg, 1.04 mmol) was stirred in 15 mL 1*N* H_2SO_4 at 40 °C for 1.5 h. The volume was reduced to one-half and the product was precipitated with EtOH, filtered, washed with EtOH and dried *in vacuo* over P_2O_5 to give 459 mg (0.943 mol, 91%) of a white solid. Mp 181 °C; FABMS: 390 $(M)^+$ of cation. ^1H NMR (Me_2SO-d_6) δ 9.42 (s, 1, H-2″), 8.89 (d, 1, H-6″), 8.90 (d, 1, H-4′), 8.85 (s, 1, H-5), 8.15 (t, 1, H-5″), 7.88 and 7.63 [2 s, 2, -C(=O)-NH_2], 7.71 (d, 1, -C*H*=CHCOO), 7.02 (d, 1, -CH=C*H*COO), 5.95 (s, 1, H-1′), 5.70 and 5.45 (2 s, 2, -OH-2′, -OH-3′), 4.55-4.15 (m, 5, H-2′, H-3′, H-4′, 2 H-5′), 4.40 (s, 3, N-CH_3). ^{13}C NMR (Me_2SO-d_6) δ 164.27 [1, -C(=O)-O-], 160.32 [1, -C(=O)-NH_2], 157.37 (1, C-3), 145.58 (1, C-5), 145.52 (1, C-2″), 145.11 (1, C-6″), 142.88 (1, C-4″), 137.24 (1, -*C*H=CHCOO), 133.70 (1, C-3′), 127.47 (1, C-5″), 124.21 (1, -CH=*C*HCOO-), 91.20 (1, C-1′), 81.53 (1, C-4′), 74.05 (1, C-3′), 70.13 (1, C-2′), 63.81 (1, C-5′), 48.07 (1, N-CH_3). *Anal.* Calcd for $C_{17}H_{20}N_5O_6^+ \cdot HSO_4^-$: C, 41.88; H, 4.35; N, 14.37. Found: C, 41.82; H, 4.59; N, 14.16.

2-(2,3-O-Methylethylidene-β-D-ribofuranosyl)selenazole-4-carboxamide (32). To a suspension of selenazofurin (**31**, 5.00 g; 16.3 mmol) and 2,2-dimethoxypropane (5.0 mL, 40.65 mmol) in 25 mL of dry acetone at 0 °C was added 0.47 mL of perchloric acid (70%). After 5 min the ice bath was removed and the solution was allowed to warm to room temperature. After 45 min the solution was neutralized with 1*N* NaOH and the solvent removed at room temperature under reduced pressure. The crude product was purified by plug filtration over silica gel (EtOAc-EtOH, 95:5), then recrystallized from EtOAc-cyclohexane to give 5.20 g of a white crystalline solid suitable for use (14.99 mmol; 92%). FABMS: 349 $(M + 1)^+$. ^1H NMR (Me_2SO-d_6) δ 8.84 (s, 1, H-5′), 7.76 and 7.56 (2 s, 2, -C=O-NH_2), 5.08 (d, 1, H-1′), 5.02-4.95 (m, 2, OH-5′ and H-2′), 4.74 (dd, 1, H-3′), 4.15 (m, 1, H-4′), 3.55-3.35 (2 m, 2, H-5a′ and H-5b′), 1.52 and 1.32 (2 s, 6, $C(CH_3)_2$.

2-[2,3-O-Methylethylidene-5-O-(3-pyridinylcarbonyl)-β-D-ribofuranosyl]selenazole-4-carboxamide (33). A suspension of **32** (4.23 g, 0.012 mol) nicotinic acid (1.69 g, 0.014 mol), DMAP (1.66 g, 0.014 mol) and DCC (2.80 g, 0.014 mol) in 50 mL of dry THF was stirred for 16 h at room temperature. The solid that separated was filtered off and washed, and the solvent was removed under reduced pressure. The product was purified by flash chromatography ($CHCl_3$-MeOH, 97:3) to give 4.46 g (0.010 mol; 82%) of a white foam. FABMS: 454 $(M + 1)^+$. ^1H NMR ($CDCl_3$) δ 9.12 (d, 1, H-2''), 8.78 (dd, 1, H-6''), 8.76 (s, 1, H-5), 8.12 (dd, 1, H-4''), 7.38 (m, 1, H-5''), 7.18 and 5.85 (2 s, 2, -C=NH_2), 5.20 (d, 1, H-1'), 5.08 (m, 1, H-2'), 4.85 (dd, 1, H-3'), 4.66 (d, 1, H-4'), 4.52 (m, 1, H-5a'), 4.45 (m, 1, H-5b'), 1.65 and 1.44 (2 s, 6, $C(CH_3)_2$). UV λ_{max} 261 (pH 1.0), 263 (pH 7.0), 262 (pH 13).

2-[5-O-(1-Methyl-3-pyridiniumcarbonyl)-β-D-ribofuranosyl]selenzole-4-carboxamide Iodide (34). A solution of **33** (3.45 g, 7.63 mmol) and iodomethane (2.8 mL, 44 mmol) in 200 mL of acetone was heated at 70 °C for 36 h. The solution was allowed to cool and the pyridinium salt was precipitated by the addition of 900 mL of ether to give 3.85 g of a yellow solid [FABMS 468 $(M)^+$ of cation]. The solid was then dissolved in 1*N* H_2SO_4-dioxane (13 mL/30 mL) and stirred overnight at room temperature. The solution was concentrated to one-half volume and the product precipitated by the addition of ethanol to give 2.66 g (5.05 mmol; 66%) of an orange-yellow solid. FABMS 428 $(M)^+$ of cation. ^1H NMR (Me_2SO-d_6) δ 9.52 (s, 1, H-2''), 9.20 (d, 1, H-6''), 8.95 (d, 1, H-4''), 8.75 (s, 1, H-5), 8.35 (dd, 1, H-5''), 7.64 and 7.58 (2 s, 2, -C=O-NH_2), 5.58 (s, 1, -OH-2'), 5.35 (s, 1, -OH-3'), 4.94 (d, 1, H-1'), 4.78 (dd, 1, H-5a'), 4.54 (dd, 1, H-5b'), 4.44 (s, 3, N^+-CH_3), 4.28 (m, 1, H-4'), 4.21 (s, 1, H-2'), 4.10 (d, 1, H-3').

2-[5-O-(1-Methyl-1,4-dihydro-3-pyridinylcarbonyl)-β-D-ribofuranosyl]selenazole-4-carboxamide (35). To a solution of **34** (370 mg, 0.703 mmol) and sodium bicarbonate (234 mg, 2.79 mmol) in 15 mL of degassed water was added sodium dithionite (300 mg, 1.72 mmol). The product oiled out at 3 min and the mixture was stirred an additional 7 min. The solution was then decanted from the oil and applied to a BioBead column with water. After initial washing with water, the product was eluted with methanol. The eluted product and oil were combined and lyophilyzed from water to give 154 mg of a yellow solid. FABMS: 430 $(M + 1)^+$ of the dihydro compound; 428 $(M)^+$ of cation. TLC analysis ($CHCl_3$-MeOH, 3:1) and HPLC studies revealed the product to be contaminated with selenazofurin (**30**). Attempts to purify by flash chromatography were unsuccessful. Further TLC studies revealed that the prodrug hydrolyzes readily on the TLC plates. Similar results are obtained by HPLC analysis in buffered systems. The ^1H NMR is a complex mixture of the dihydro prodrug and its hydrolysis products.

III. PHARMACOLOGY

PHARMACOKINETICS OF THE BODOR CONJUGATE OF RIBAVIRIN (SRI-6711) IN MICE

INTRODUCTION

The purpose of these studies was to determine disposition of [^{14}C]SRI-6711 in male Swiss mice following i.v. administration.

MATERIALS AND METHODS

Dose Solution Preparation. Unlabeled and [^{14}C]-labeled SRI-6711 were dissolved in dimethylacetamide and then diluted with water to yield a solution containing 5 mg/ml SRI-6711 and having a specific activity of approximately 4.4 μCi/mg. By HPLC analysis, 84% of the radioactivity in the dosing solution was present as the reduced form of the prodrug ([^{14}C]SRI-6711), and 16% as [^{14}C]ribavirin.

Dosing and Sample Collection. Female CFW (Swiss) mice, weighing about 25 g, were administered 50 mg/kg [^{14}C]SRI-6711 (about 6 μCi), i.v. At selected times after dosing, mice were anesthetized with diethyl ether; and a single, terminal blood sample was collected from the axillary region into a tube containing heparin. Plasma was obtained after centrifugation of cooled samples. Various tissues were removed separately and immediately frozen on dry ice. For the collection of urine, mice were maintained in glass metabolism cages.

Sample Analysis. Plasma and urine were assayed for total radioactivity after the addition of Safety Solve counting cocktail. Tissues were homogenized in 2 vol. of water and portions of each homogenate were radioassayed after solubilization in Soluene 350 tissue solubilizer. Prior to HPLC analysis, plasma and tissue homogenates were extracted with 4 vol. of acetonitrile. Some samples were concentrated by evaporation under nitrogen prior to assay.

Separation of ribavirin and the oxidized and reduced forms of the prodrug was achieved by HPLC using a Whatman ODS-2 column and a 20 min linear gradient from 25 mM $NH_4H_2PO_4$ (pH 4.5) to 25 mM $NH_4H_2PO_4$ (pH 4.5):CH_3CN (87.5:12.5; v,v) at 1 ml/min (**Figure 5**). Elution at final conditions was continued for 20 min. The column was equilibrated at initial conditions for 30 min between analyses. One minute fractions at eluting material were collected for radioanalysis.

RESULTS

Following i.v. administration, [^{14}C]SRI-6711 was rapidly eliminated from plasma with a half-life of <5 min (**Figure 6**). The disappearance of the reduced prodrug was associated with a progressive increase in the percentage of radioactivity present as [^{14}C]ribavirin; within 60 min, this compound accounted for essentially all of the radioactivity in plasma (**Table 1**). In contrast, the oxidized prodrug was detectable in only one sample obtained at 30 min (**Table 1**). Total radioactivity was eliminated from plasma in an initial phase of about 20 min and then in a prolonged phase of >9 hr (**Figure 6**). The levels of radioactivity in plasma samples obtained beyond 1 hr were too low to permit analyses by HPLC.

As noted above, 16% of the radioactivity in the dosing solution was present as [^{14}C]ribavirin. Each mouse, therefore, received approximately 200 μg of [^{14}C]ribavirin as an impurity in the dose formulation. Assuming a volume of distribution of 20 ml for a 25 g mouse, this amount of compound could have produced initial plasma levels of [^{14}C]ribavirin of about 10 μg/ml. The influence of this on the results obtained during this study cannot be deduced from the data.

The results of the radioanalyses of brain are shown in **Figures 5** and **7** and **Tables 1** and **2**. Little radioactivity (<0.05% of the dose) was detected in this tissue at any time after dosing. At 15 and 30 min, both the reduced and oxidized forms of the prodrug, as well as ribavirin, were present at very low levels. Beyond this time, all radioactivity in brain was associated with [^{14}C]ribavirin.

Analyses of other tissues demonstrated that highest levels of radioactivity were in liver, kidney and spleen (**Figure 7**). With the exception of liver samples obtained at 15 and 30 min, essentially all the radioactivity in these tissues was [^{14}C]ribavirin (**Table 1**). Due to relatively low levels of radioactivity, spleen, muscle, lung and fat were not assayed by HPLC.

In three mice, 53% (SD ± 3) of the administered dose was excreted into urine within 24 hr. HPLC analyses of two urine samples indicated that, of the radioactivity recovered in urine, about 34, 20, and 47% was present as unchanged [^{14}C]SRI-6711, the oxidized form of [^{14}C]SRI-6711, and [^{14}C]ribavirin, respectively (**Table 1**).

SUMMARY AND CONCLUSIONS

The results of this study demonstrate that unchanged [^{14}C]SRI-6711 was rapidly eliminated from plasma following i.v. administration. In contrast, a prolonged phase of elimination of total radioactivity from plasma was observed. In tissues, highest levels of radioactivity were in liver, kidney and spleen; most of the radioactivity in these tissues was present as [^{14}C]ribavirin. Lowest levels (<0.05% of the dose) of radioactivity were in brain and fat. These data indicate that only very low levels of either [^{14}C]SRI-6711 or its radiolabeled metabolic products are transported into the brain following administration of [^{14}C]SRI-6711.

TABLE 1

HPLC ANALYSES OF PLASMA, URINE AND TISSUE EXTRACTS OBTAINED FROM MICE ADMINISTERED 50 MG/KG OF [^{14}C]SRI-6711, I.V.

Tissue	Sample Time	Mouse No.	µg equiv./ ml or g of Sample	% Extractable ^{14}C Present as: Ribavirin	Oxidized Prodrug	Reduced Prodrug
Plasma	15 min	A	22.0	26		74
		B	25.8	73		27
		C	18.3	33		67
	30 min	A	11.4	54	35	10
		B	7.5	94		6
	60 min	B[a]	5.8	87		13
		C[a]	5.9	100		
Brain[a]	15 min	A,B,C[b]	1.6	44	14	41
	30 min	A,B	2.0	52	35	13
	60 min	A	1.0	100		
		B	1.0	100		
		C	0.7	100		
	3 hr	A,B,C	1.5	100		
	6 hr	A,B,C	0.9	100		
Liver	15 min	A,B,C	123.5	51		49
	30 min	A,B	81.6	75		25
	1 hr	A,B,C	65.9	100		
	3 hr	A,B,C	28.2	100		
Kidney	15 min	A,B,C	53.7	98		2
	30 min	A,B	33.8	100		
	1 hr	A,B,C	26.6	100		
Spleen	15 min	A,B,C	20.4	100		
	30 min	A,B	48.6	100		
	1 hr	A,B,C	33.1	100		
Urine	24 hr	A	184.5	48	17	35
	24 hr	B	151.5	45	22	33

[a]Extracts were concentrated prior to HPLC analysis.

[b]Tissues from individual mice were pooled prior to analysis.

TABLE 2

LEVELS OF RADIOACTIVITY IN MOUSE BRAIN AT VARIOUS TIMES FOLLOWING I.V. ADMINISTRATION OF 50 MG/KG [^{14}C]SRI-6711

Time	Mouse No.	Dose (μCi/mouse)	nCi/brain	% Dose in brain
15 min	A	5.77	1.93	0.03
	B	5.77	1.38	0.02
	C	5.77	0.85	0.01
30 min	A	5.99	1.28	0.02
	B	5.77	1.76	0.03
60 min	B	5.99	1.52	0.03
	C	5.77	1.23	0.02
3 hr	A	5.55	1.03	0.02
	B	5.77	0.80	0.01
	C	5.77	1.06	0.02
24 hr	A	5.99	0.70	0.01
	C	5.77	0.65	0.01

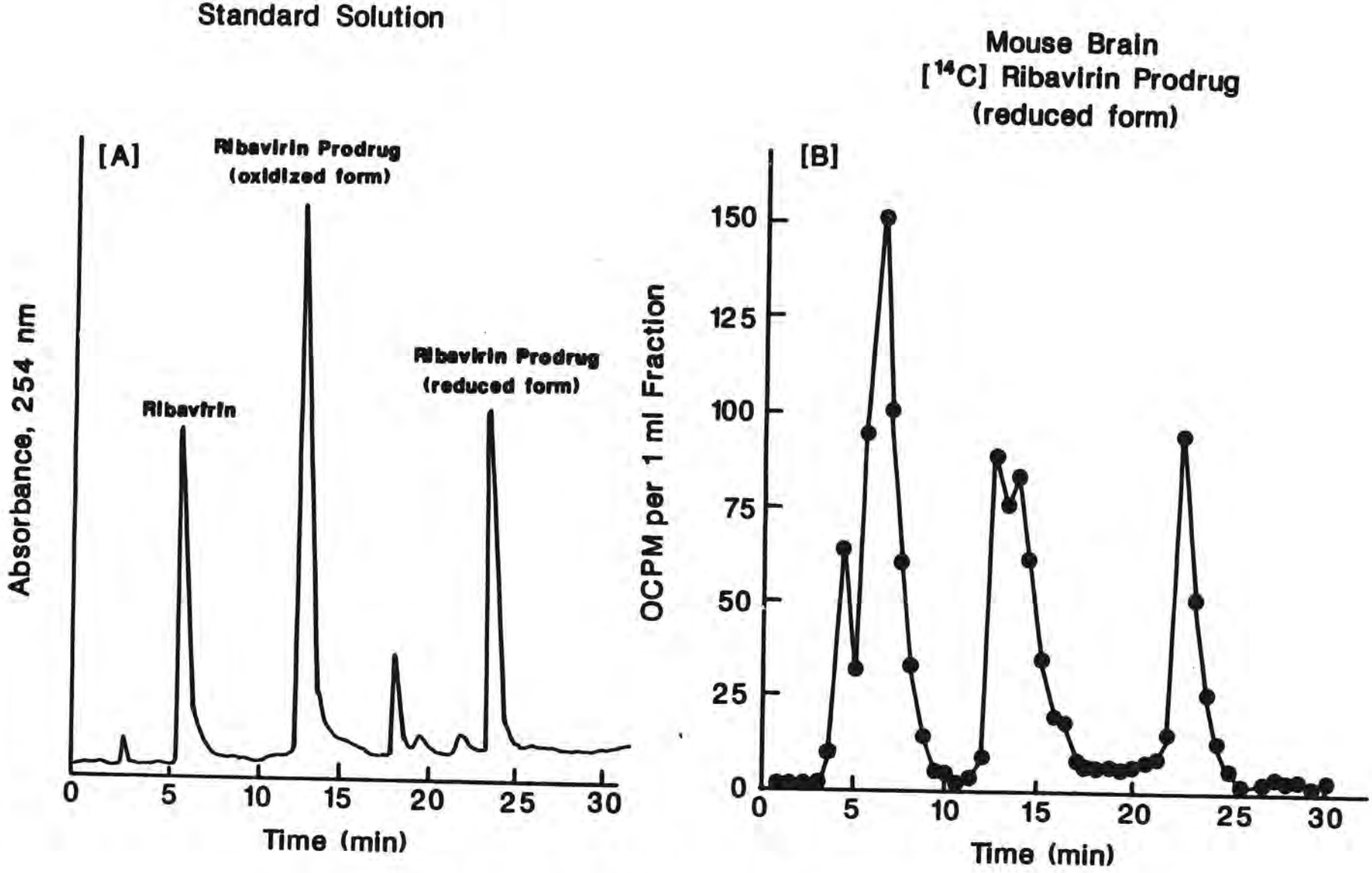


Figure 5 HPLC analyses of [A] a standard solution containing the reduced and oxidized forms of SRI 6711 (ribavirin prodrug) and ribavirin; [B] an extract of mouse brain obtained 30 min after administration of 50 mg/kg of [^{14}C] SRI-6711.

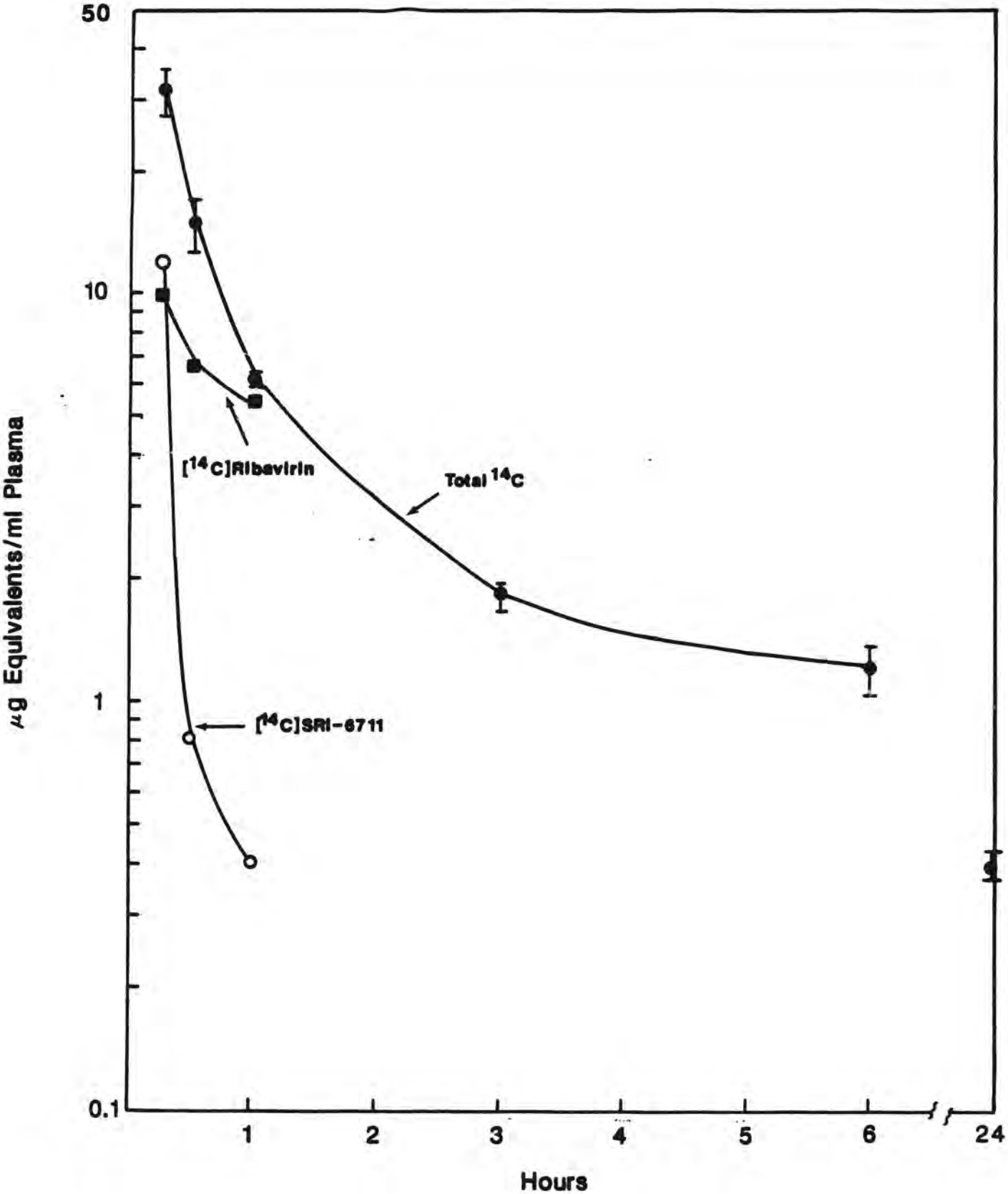


Figure 6 Plasma elimination of total radioactivity (●), [^{14}C] SRI-6711 (O) and [^{14}C] ribavirin (■) following i.v. administration of [^{14}C] SRI-6711 to mice.

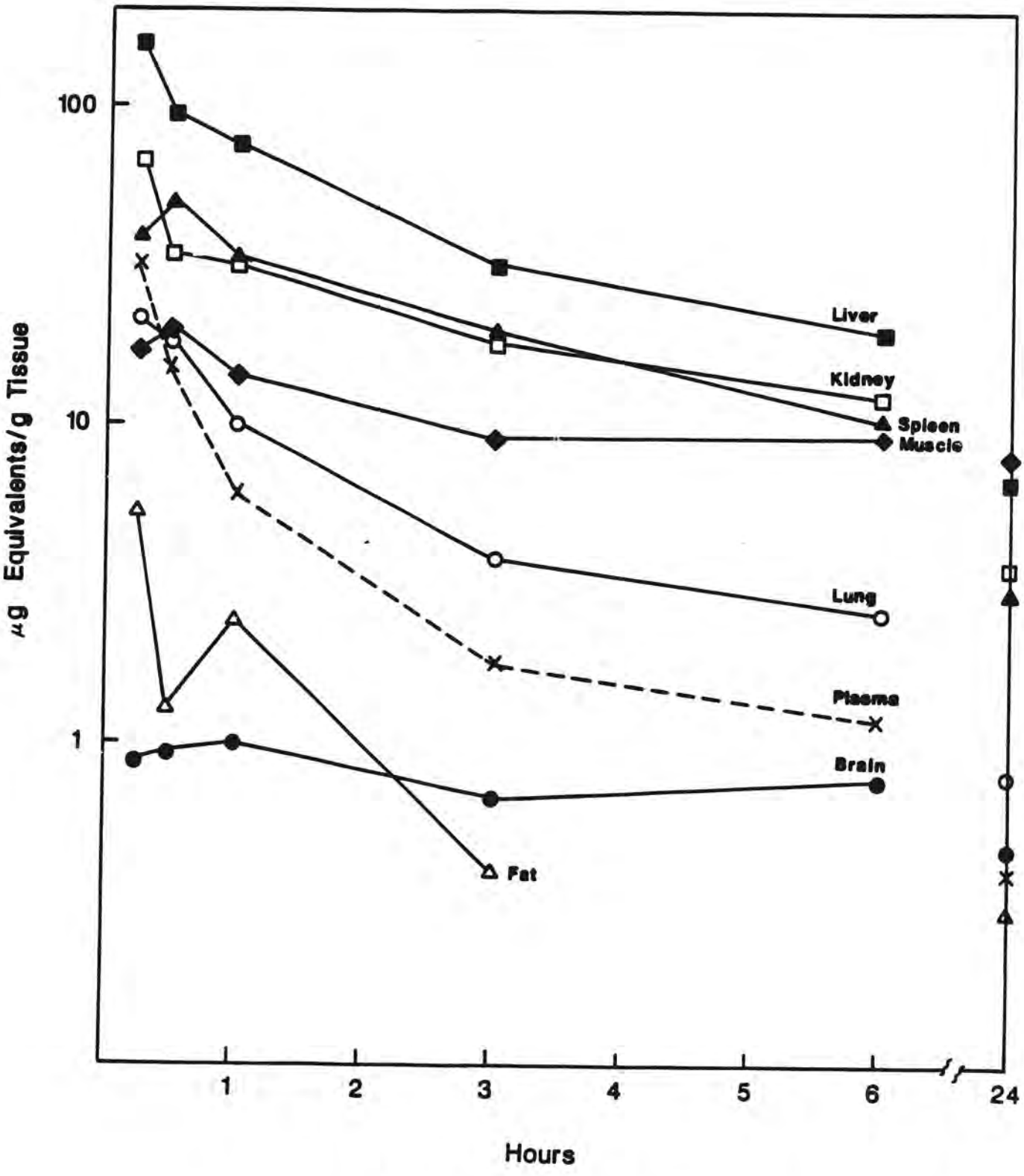


Figure 7 Tissue levels of total radioactivity following i.v. administration of [^{14}C] SRI-6711 to mice.

IV. VIROLOGY

A. Preliminary Study

This research project involved the synthesis, pharmacology, and *in vivo* antiviral evaluation of certain prodrug esters of ribavirin and of other antiviral drugs designed to pass the blood-brain barrier in sufficient quantities to inhibit the replication of viruses of the Togaviridae family. Ribavirin prodrugs, synthesized in this laboratory were evaluated for therapeutic efficacy against Japanese encephalitis (JE) virus and Venezuelan equine encephalitis (VEE) virus infections in mice.

A sample of our initial dihydropyridine conjugate of ribavirin was submitted to the Contracting Officer's Technical Representative (Dr. Michael A. Ussery) at Fort Detrick, Frederick, MD for preliminary antiviral evaluation for therapeutic efficacy in the treatment of lethal JE virus infections in mice. C57Bl/6 mice (ten animals per group) were inoculated with either 10 or 1000 LD_{50} of JE virus (Peking strain) and treated with either ribavirin, ribavirin prodrug, or (for placebo-treated controls) with drug vehicle alone. The ribavirin prodrug was administered to mice by the intraperitoneal (IP) route at a dose level of 45 mg/kg/day once a day for nine days, beginning on Day -1 (preinfection) and ending on Day +7 (post-infection). The parent drug was administered to mice by the same route and on the same schedule at a dose level of 50 mg/kg/day. There were no survivors at the 1000 LD_{50} virus challenge level. Among the animals challenged with 10 LD_{50} of virus, however, the data indicate that there was a 40% survival rate in the ribavirin prodrug-treated group. A 100% mortality rate was observed in both the ribavirin- and the placebo-treated groups. No significant increases in mean survival time of dying animals were observed in the treated groups when compared to virus-infected controls. These preliminary data indicated that the ribavirin prodrug crossed the blood-brain barrier and produced a significant therapeutic effect in this animal model system. The parent drug, ribavirin, was without effect in protecting mice from lethal viral encephalitis.

During the first year of the contract, much effort was expended in renovating and equipping the SRI-UAB P-3 facility, recruiting and training staff members in P-3 containment procedures, and in completing our immunization program for P-3 facility personnel. In August, 1986, building renovations were completed and the facility was certified as a Biosafety Level-3 (BL-3) containment facility. Full approval was subsequently given for appropriately immunized personnel to commence work with BL-3 level viruses.

B. *In Vitro* Culture of Japanese Encephalitis-B Virus and Venezuelan Equine Encephalomyelitis Virus

The Nakayama strain of Japanese encephalitis-B virus (JE) and the Trinidad donkey strain of Venezuelan equine encephalitis (VEE) virus, obtained from Dr. George R. French (Salk Institute, Swiftwater, PA) were used to prepare stocks of virus. Seed stocks of the JE virus and of the VEE virus were prepared by infection of Vero cell cultures at an input multiplicity of infection of 0.01 to 0.1. From these seed stocks, working stocks (second passage) of each of the viruses were produced in Vero cell cultures. Each working stock was titrated by plaque and end-point CPE assays in Vero cell cultures. Working stocks of each virus were prepared in large volume, titrated and frozen for *in vivo* studies.

Because the Nakayama strain of JE virus was found to be inappropriate for *in vivo* studies, the Beijing strain of JE virus was obtained from Major M. Ussery (USAMRIID) for use in an *in vivo* challenge model. A seed stock of this virus was prepared in Vero cells. This seed stock was used to prepare a working stock (second passage) which was titrated and frozen for use in the *in vivo* challenge studies.

C. *In Vivo* Work with JE Virus, Nakayama Strain

The initial *in vivo* challenge experiments with JE virus, Nakayama strain, were conducted in outbred 20-25 gm female Swiss mice (CD-1 virus antibody fee (VAF+); Charles River Labs). Groups of mice were challenged by the IP or IC route with virus inocula ranging from 10,000 to 0.75 pfu/mouse. Virus challenge with as few as 10 pfu administered intracranially was uniformly fatal. The results of virus challenge

administered intraperitoneally were variable in that no linear relationship between the viral challenge and the percent mortality occurred (Average Day of Death [ADD]:10 - 18 days).

Because of the inconsistent mortality in outbred CD-1 mice following IP challenge, the potential value of inbred C57Bl/6 mice was evaluated in challenge experiments using the Nakayama strain of JE virus. Age and sex matched CD-1 and C57Bl/6 mice were challenged by the IP or IC routes with the same virus preparations at levels ranging from 250 to 0.75 pfu/mouse. Both mouse strains suffered 100% mortality following IC challenge with 1.5 to 250 pfu. The results of IC challenge with a theoretical 0.75 pfu/mouse indicated that the LD_{50} was in the range of 1 pfu/mouse. The average day of death following IC challenge was not significantly different for the two strains of mice. The results of IP challenge in both strains were variable with a total of 25% mortality occurring in the challenged animals of each strain. The ADD occurred over a broader range for the CD-1 mice (11 - 17 days) than for the C57Bl/6 mice (15-16 days). No advantage in using C57Bl/6 mice rather than CD-1 mice was identified when the Nakayama strain of JE virus is used. The cumulative data generated in JE challenge experiments using the Nakayama strain of virus indicated that the LD_{90} for second passage JE virus administered by the IC route was approximately 1.1 pfu/mouse. In an attempt to generate a pool of the Nakayama strain of JE virus which was lethal to CD-1 mice by the IP route, the virus was serially passaged in CD-1 mice. However, the virus preparations generated by *in vivo* passage only resulted in consistent mortality in animals challenged by the IC route. No enhancement of IP virulence was obtained by serial *in vivo* passage.

D. *In Vivo* Work with JE Virus, Beijing Strain

The Beijing strain of JE virus was titrated in CD-1 female mice by the IP challenge route. The mortality obtained with this strain of virus following IP challenge in CD-1 mice was extremely variable and did not provide a viable model. A series of *in vivo* passages of this virus were performed in C57Bl/6 mice to enhance the intraperitoneal pathogenicity. The virus was then titrated in C57Bl/6 mice which are more sensitive to its lethal effects. Preparations of JE virus which were lethal following IP challenge in C57Bl/6 mice were obtained.

E. *In Vivo* Work with VEE Virus

The second passage of VEE virus, Trinidad Donkey strain, was titrated in CD-1 female mice by the IP and IC challenge routes. The LD_{90} for VEE administered IC is 0.16 pfu/mouse while the LD_{90} for the IP inoculated virus is 1.3 pfu/mouse. The ADD for IP challenged animals ranged from 7.6 days to 11.8 days. The ADD for IC challenged animals ranged from 6.2 days to 8.5 days. The LD_{50} is less than 1 pfu/mouse which may be due to difference in *in vitro* and *in vivo* virulence. A similar effect was reported by Taylor *et al.* in JE virus challenge studies performed in mice.

F. *In Vivo* Antiviral Studies with Japanese Encephalitis-B Virus and Venezuelan Equine Encephalomyelitis Virus

1. General protocol for *in vivo* assays

The virus is diluted in Modified Eagle's Medium (MEM) supplemented with 2% heat-inactivated fetal calf serum. The virus preparations are held on ice during preparation and inoculation to reduce inadvertent inactivation. Virus for intraperitoneal (IP) inoculation is prepared in an inoculum volume of 0.5 ml while virus for intracerebral (IC) inoculation is prepared in an inoculum volume of 0.03 ml. During *in vivo* virus titrations, drug treatment stress is simulated by treating mice subcutaneously (SC) with 0.9% saline (0.1 ml/10 gm body wt) q24hr for a total of 7 injections. Animals are monitored for a period of 21 days post-infection and body weights are recorded daily on days 0 through 7 and weekly thereafter.

2. Evaluation of the initial ribavirin prodrug in Venezuelan Equine Encephalomyelitis (VEE) virus infected mice

The Bodor-type, 1,4-dihydropyridyl prodrug ester of ribavirin was evaluated for antiviral efficacy in CD-1 female mice which had been challenged IP with VEE virus. For comparison, the parent compound,

ribavirin, was assessed in parallel. Both compounds were administered intraperitoneally at dose levels of 25, 50 or 100 mg/kg/day on a qd 1-7 schedule using an injection volume of 0.1 ml per 1 gram of body weight. Controls included untreated, virus-infected mice, sham-treated, virus-infected mice, sham-treated uninfected mice and normal uninfected mice. All animals were observed for a 21-day period after virus challenge. The results, presented in **Table 3**, indicate that treatment of VEE-infected mice with either ribavirin or the Bodor-type prodrug ester of ribavirin was ineffective in preventing virus-induced mortality or in increasing the mean survival time of virus-infected animals.

Table 3

Evaluation of a Bodor-Type Prodrug of 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin) for Antiviral Efficacy Against Venezuelan Equine Encephalitis (VEE) Virus in Random-Bred (CD-1) Swiss Mice

		Day 21 Mortality		
	Drug Dose	Uninfected	Infected	
Treatment Group	mg/kg	No. Surv./Total	No.Surv./Total	MDD[a] + S.D.
Untreated controls	0	0/10	8/10	8.9 ± 1.6
Sham-treated (PBS) controls	0	0/10	19/19	7.8 ± 1.6
Ribavirin prodrug	100	0/4	9/9	7.1 ± 1.1
Ribavirin prodrug	50	0/5	10/10	7.7 ± 1.3
Ribavirin prodrug	25	0/4	10/10	7.3 ± 1.6
Ribavirin	100	0/5	10/10	7.0 ± 2.2
	50	0/5	9/10	6.3 ± 1.2
	25	0/5	10/10	7.2 ± 1.6
Virus diluent control	0	0/10	-	NA

[a]MDD = Mean day of death ± standard deviation.

3. Evaluation of the initial ribavirin prodrug in Japanese Encephalitis-B virus infected mice

The Bodor-type, 1,4-dihydropyridyl prodrug ester of ribavirin was evaluated for antiviral efficacy in CD-1 female mice which had been challenged intracranially with JE (Nakayama strain) virus. For comparison the parent compound, ribavirin, was assessed in parallel. Both compounds were administered intraperitoneally at dose levels of 50 mg/kg/day on a qd 1-7 schedule using an injection volume of 0.1 ml per 1 gram of body weight. Controls included untreated, virus-infected mice, sham-treated, virus-infected mice, sham-treated uninfected mice and normal uninfected mice. All animals were observed for a 14-day period after virus challenge. The results, presented in **Table 4**, indicate that treatment of intracranially challenged JE infected mice with either ribavirin or the Bodor-type prodrug ester of ribavirin was ineffective in preventing virus-induced mortality or in increasing the mean survival time of virus-infected animals.

Table 4

Results of Prodrug Activity in JE Virus Infected Mice

Treatment[a]	No. Uninfected	No. Infected
Untreated	0/5[b]	10/10 (7.8 ± 2.1)[c]
PBS x 7 d IP	0/5	10/10 (6.4 ± 0.5)
50 mg/kg prodrug IP	0/5	10/10 (7.3 ± 2.1)
50 mg/kg ribavirin IP	0/5	10/10 (7.7 ± 2.4)
Sham-infected	0/5	NA

[a]The mice were challenged intracranially with 5 pfu of JE virus, Nakayama strain. Therapy was initiated 4 hours post-infection on a qd 1-7 treatment schedule.

[b]No. dead animals/No. treated animals.

[c]Average day of death ± the standard deviation.

4. Antiviral evaluation of SRI 6711, SRI 7222 and SRI 7223

Female C57Bl/6 mice (VAF+, Charles River Labs) weighing 14-16 gms were randomly divided into treatment groups consisting of 15 mice each. The mice were treated intraperitoneally once daily for 9 days with one of the following (1) 10% DMSO in water, (2) 75 mg/kg SRI 6711, (3) 60 mg/kg SRI 7222, (4) 50 mg/kg SRI 7223, (5) 50 mg/kg ribavirin, (6) 25 mg/kg ribavirin or (7) 35 mg/kg selenazole. Compound treatments began the day preceding virus challenge. Compounds were administered two hours prior to challenge on the day of virus inoculation. Compounds were prepared immediately before use by dissolving in 1 part 90% DMSO and diluting with 9 parts sterile water. Five of the mice were challenged intraperitoneally with 0.5 ml of a 1:8000 dilution of JEV Challenge Prep #4 (approximately 9 LD_{50}). The remaining five mice were challenged intraperitoneally with 0.5 ml of a 1:6000 dilution of JEV Challenge Prep #4 (approximately 4.5 LD_{50}). Untreated, infected and untreated, uninfected animals were included as controls. As shown in **Table 5**, no reduction in mortality occurred in any treatment group although there was some prolongation of the average day of death (ADD) in animals receiving SRI 6711 and challenged with a 1:16,000 dilution of the virus prep. The ADD of the mice receiving 75 mg/kg SRI 6711 was 14.8 days while the diluent-treated animals had an ADD of 11.9 days ($p = .02$). Although there was no reduction in the mortality for any treatment group the prolongation in ADD for SRI 6711 treated animals receiving the lower virus challenge implies that this compound may be effective if higher doses are administered for a longer treatment schedule and if the virus challenge is not excessive.

Table 5

Antiviral Evaluation of SRI 6711, SRI 7222 and SRI 7223

	No. dead/ No. Uninfected	No. dead/[a] No. Challenged (ADD)[c]	No. dead/[b] No. Challenged (ADD)[c]
untreated	0/5	10/10 (11.6 ± 2.6)	10/10 (12.5 ± 1.3)
10% DMSO/water	0/5	9/10 (12.7 ± 1.8)	10/10 (11.9 ± 2.1)
75 mg/kg SRI 6711	0/5	5/5 (12 ± 1.2)	5/5 (14.8 ± 1.6)[d]
60 mg/kg SRI 7222	0/5	5/5 (11.6 ± 3.1)	5/5 (13.2 ± 1.8)
50 mg/kg SRI 7223	0/5	5/5 (11.6 ± 1.1)	5/5 (12.2 ± 0.4)
50 mg/kg ribavirin	0/5	5/5 (13.6 ± 2.4)	5/5 (12.0 ± 1.2)
25 mg/kg ribavirin	0/5	5/5 (12.4 ± 1.8)	5/5 (13.0 ± 2.5)
35 mg/kg AVS253	0/5	5/5 (11.8 ± 0.4)	5/5 (11.6 ± 0.9)
1:5000 dilution challenge virus		10/10 (12.6 ± 2.3)	
1:8000 dilution challenge virus		10/10 (11.7 ± 1.3)	
1:10,000 dilution challenge virus		9/10 (12.7 ± 1.0)	
1:16,000 dilution challenge virus		10/10 (12.6 ± 1.4)	
1:20,000 dilution challenge virus		10/10 (12.2 ± 2.0)	
1:25,000 dilution challenge virus		10/10 (12.9 ± 1.4)	
1:30,000 dilution challenge virus		10/10 (12.8 ± 1.0)	
1:32,000 dilution challenge virus		9/9 (13.3 ± 1.7)	
1:40,000 dilution challenge virus		10/10 (12.1 ± 2.9)	
1:64,000 dilution challenge virus		8/10 (12.3 ± 2.6)	

[a]challenged intraperitoneally with 0.5 ml of 1:8000 dilution of virus prep

[b]challenged intraperitoneally with 0.5 ml of 1:16,000 dilution of virus prep

[c]ADD average day of death ± 1 Standard Deviation

[d]Student's t-test $p = 0.02$

G. Alternative In Vivo Virus Challenge Models

1. Punta Toro Virus

In order to better evaluate drugs designed to cross the blood-brain barrier, especially ribavirin and selenazole derivatives, development of a new virus model was initiated. This model involves the use of intracerebrally (i.c.) inoculated Punta Toro virus (strain Balliet), because of its sensitivity to ribavirin. This was done because neither ribavirin nor the new derivatives synthesized at SRI which were designed to cross the blood-brain barrier were active against intraperitoneally (IP) inoculated VEE virus. This data seemed to reflect the fact that ribavirin is minimally active against VEE in vitro.

The Balliet strain of PT was purchased from ATCC. A brain pool was prepared by passaging the virus in C57Bl/6 mice after intracerebral (i.c.) inoculation. An initial i.c. titration was performed (**Table 6**) and the LD_{90} appeared to be at $\approx$ the 10^{-4} dilution.

Prior to performing an experiment, we administered ribavirin intracerebrally to mice in doses of 40 mg/kg and greater. It seemed from these experiments, that 40 mg/kg would be an appropriate high dose to use in an experiment. Therefore, an experiment using 4 week old C57Bl/6 mice was planned with treatment times of 4, 6, 8 or 10 hours post-virus inoculation. The mice were randomly assigned to the following experimental groups:

1. PBS, 4 hr post-infection
2. PBS, 6 hr post-infection
3. PBS 8 hr post-infection
4. PBS, 10 hr post-infection
5. 40 mg/kg AVS 01, 4 hr post-infection
6. 40 mg/kg AVS 01, 6 hr post-infection
7. 40 mg/kg AVS 01, 8 hr post infection
8. 30 mg/kg AVS 01, 4 hr post-infection
9. 30 mg/kg AVS 01, 6 hr post-infection
10. 30 mg/kg AVS 01, 8 hr post-infection
11. 20 mg/kg AVS 01, 10 hr post-infection
12. 10 mg/kg AVS 01, 10 hr post-infection

The results, shown in **Table 7**, indicate that a dose of 40 mg/kg of ribavirin was too high for i.c. administration. Even the 30 mg/kg dose was toxic (death immediately followed treatment) to 50 or 60% of the animals. In groups treated with 30 mg/kg AVS 01, the increase in average day of death (ADD) was impressive with p values by the Student's t test from 0.015 to 0.054. At the 10 hour treatment time, the drug dosage was reduced to 20 and 10 mg/kg. For these dosages, the ADD was increased by about 2 days with significant p values of 0.0039 and 0.016 being calculated. This experiment suggested that target organ therapy of Punta Toro Virus infections was possible. Therefore, we performed an additional experiment, using 20 and 10 mg/kg ribavirin administered 8 hr post-infection (**Table 8**). In this experiment, d[illegible] treatment at 8 hr post-infection, also produced significant increases in ADD (2-2.5 days). These data suggest that a target organ system can be established with Punta Toro virus, which would allow the evaluation of compounds for which only small quantities are available. Also, this method would allow determination of potential brain activity of drugs for which structural modification to enhance brain deliver might be pursued.

Table 6

Initial Intracerebral and Subcutaneous Titration of Punta Toro (Balliet) in C57Bl/6 Mice

Group	No. Challenged	ADD[a] ± 1 SD
$10^{-1.0}$ IC	10/10	6.2 ± 0.9
$10^{-1.5}$ IC	10/10	7.1 ± 0.9
$10^{-2.0}$ IC	10/10	7.7 ± 1.3
$10^{-2.5}$ IC	10/10	8.2 ± 1.0
$10^{-3.0}$ IC	10/10	8.7 ± 1.0
$10^{-3.5}$ IC	10/10	9.1 ± 0.9
$10^{-4.0}$ IC	8/10	10.1 ± 1.1
$10^{-5.0}$ IC	1/10	12.0 ± 0.0

[1]Add ± 1 SD = Average Day of Death ± 1 Standard Deviation

$$\text{ADD} = \frac{\Sigma[(\text{day of death}) \times (\text{number of dead that day})]}{\text{Total Number Dead}}$$

[b]N/A - Not applicable

Table 7

Effect of Ribavirin on Punta Toro Virus Infection of C57Bl/6 Mice Following Intracerebral Virus Inoculation and Treatment

	No Dead / No. Uninfected	No. Toxicity Deaths / No. Infected	No. Infectious Deaths / No. Infected	ADD ± 1 SD[1]	Probability[2]
4 hr Post-infection					
PBS[3]	ND[5]	0/10	9/10	8.33 ± 1.0	
40 mg/kg AVS 01	ND	7/10	2/3	10.00 ± 2.83	0.144
30 mg/kg AVS 01	ND	5/10	4/5	10.75 ± 3.10	0.0501
6 hr Post-infection					
PBS	ND	0/10	9/10	8.67 ± 1.32	
40 mg/kg AVS 01	ND	9/10	0/1		
30 mg/kg AVS 01	ND	6/10	4/4	11.00 ± 1.41	0.0150
8 hr Post-infection					
PBS	0/4	0/10	10/10	8.10 ± 1.52	
40 mg/kg AVS 01	4/5	7/10	3/3	9.67 ± 0.58	0.117
30 mg/kg AVS 01	3/5	5/10	5/5	10.00 ± 1.87	0.054
10 hr Post-infection					
PBS	ND	0/10	9/10	7.78 ± 1.20	
20 mg/kg AVS 01	ND	2/9	6/7	10.33 ± 1.63	0.0039
10 mg/kg AVS 01	ND	0/10	8/10	9.50 ± 1.41	0.016
8 hr Post-infection					
HBSS[4]		0/19	17/19	8.7 ± 1.30	
20 mg/kg AVS 01		0/10	6/10	11.33 ± 1.97	0.00093
10 mg/kg AVS 01		0/9	8/9	10.88 ± 2.36	0.00518

[1]Add ± 1 SD = Average Day of Death ± 1 Standard Deviation

ADD = $\frac{\Sigma[(\text{day of death}) \times (\text{number of dead that day})]}{\text{Total Number Dead}}$

[2]Probability by Student's t test

[3]PBS = Phosphate buffered saline

[4]Not done

Table 8

Effect of Ribavirin (20 and 10 mg/kg doses) on Punta Toro Virus Infection of C57Bl/6 Mice Following Intracerebral Virus Inoculation and Treatment

	No. Toxicity Deaths / No. treated	No. Infectious Deaths / No. Infected	ADD ± 1 SD[1]	Probability[2]
8 hr Post-infection				
HBSS[3]	0/19	17/19	8.7 ± 1.30	
20 mg/kg AVS 01	0/10	6/10	11.33 ± 1.97	0.00093
10 mg/kg AVS 01	0/9	8/9	10.88 ± 2.36	0.00518

[1]Add ± 1 SD = Average Day of Death ± 1 Standard Deviation

$$ADD = \frac{\Sigma[(\text{day of death}) \times (\text{number of dead that day})]}{\text{Total Number Dead}}$$

[2]Probability by Student's t test

[3]HBSS = Hank's balanced salt solution

2. VEE virus target organ model

Work was conducted in an effort to establish a target organ model for evaluating anti-VEE compounds. The VEE virus challenge stock was titrated for lethality by the intracranial route using serial dilutions of the virus stock and a standard inoculum volume of 0.03 ml. These results are shown in **Tables 9** and **10**. The intracranial LD_{90} was between the $10^{-7.4}$ and the $10^{-7.7}$ dilution of stock virus when 0.03 ml was administered. The effects of a single intracranial dose of PBS on the mortality of VEE challenged mice was assessed. In this experiment groups of 10 mice each were challenged with serial dilutions of VEE stock virus and 8 hours post-challenge they received a single dose of 0.03 ml of PBS intracranially. The results are shown in **Table 11**. Mortality occurred at all challenge levels ($10^{-7.1}$ through $10^{-8.6}$ dilutions); however, 100% mortality was present only in the $10^{-7.1}$ through $10^{-7.7}$ dilutions. The effect of a single intracranial dose of 40 mg of 3N-3DU/kg on VEE infection was assessed. Mice (10/group) were challenged intracranially with 0.03 ml of a $10^{-8.3}$ dilution of VEE stock virus and treated 6 or 8 or 10 hours later with 0.03 ml of PBS or 3N-3DU intracranially. Mice receiving 40 mg/kg 3N-3DU at 8 hr post virus challenge had a mortality rate of 25% compared to 70% in the PBS treated group. In addition, the ADD for the 3N-3DU treated group was significantly increased ($p = 0.049$). These results are shown in **Table 12**. Thus, the target organ approach may be a viable alternative for assessing compounds available in limited quantities.

Table 9

Preliminary VEE Titration

Treatment Groups	No. Dead/No. Treated	ADD ± 1 SD
Uninfected control	0/10	N/A
Sham-infected	0/10	N/A
10^{-6} dilution IC	10/10	5.9 ± 0.9
10^{-7} dilution IC	10/10	6.5 ± 0.9
10^{-8} dilution IC	5/10	6.8 ± 0.8
10^{-9} dilution IC	2/10	7.0 ± 0.0

Table 10

Secondary VEE Intracranial Challenge Titration

Treatment Groups	No. Dead/No. Treated	ADD ± 1 SD
Infected control	0/10	N/A
Sham-infected	0/10	N/A
$10^{-7.1}$ dilution IC	10/10	6.1 ± 0.3
$10^{-7.4}$ dilution IC	10/10	6.6 ± 1.2
$10^{-7.7}$ dilution IC	8/10	7.0 ± 0.8
10^{-8} dilution IC	6/10	6.7 ± 0.8
$10^{-8.3}$ dilution IC	8/10	6.4 ± 0.7
$10^{-8.6}$ dilution IC	3/9	6.7 ± 0.6
$10^{-8.9}$ dilution IC	0/10	N/A

Table 11

Mortality Following IC Challenge with VEE and Single Dose IC Treatment

Treatment Groups	No. Dead/No. Treated	ADD ± 1 SD
Uninfected, untreated	0/10	N/A
Sham-infected IC	0/9	N/A
$10^{-7.1}$ dilution of VEE, IC	10/10	5.8 ± 1.3
$10^{-7.4}$ dilution of VEE, IC	10/10	6.1 ± 0.7
$10^{-7.7}$ dilution of VEE, IC	10/10	6.5 ± 0.7
$10^{-8.0}$ dilution of VEE, IC	8/10	6.3 ± 1.3
$10^{-8.3}$ dilution of VEE, IC	4/10	7.8 ± 4.2
$10^{-8.6}$ dilution of VEE, IC	3/10	6.3 ± 0.6

Table 12

Mortality in VEE Challenged Mice Receiving a Single Dose of 3N-3DU

Treatment Groups	No. Dead/No. Treated	ADD ± 1 SD
Uninfected Mice		
Untreated control	0/5	N/A
Sham-infected IC	0/5	N/A
Sham-infected + PBS IC	0/5	N/A
Sham-infected + 40 mg/kg 3N-3DU IC	0/4	N/A
Virus-Infected Mice		
Untreated control	2/5	6.5 +/- 0.7
Placebo (PBS) 6 hrs	6/10	6.3 +/- 0.8
Placebo (PBS) 8 hrs	7/10	5.4 +/- 0.5
Placebo (PBS) 10 hrs	5/10	5.8 +/- 1.5
40 mg/kg 3N-3DU 6 hrs	6/10 (.68)	5.8 +/- 0.8
40 mg/kg 3N-3DU 8 hrs	2/8 (.077)	6.5 +/- 0.7 (.049)
40 mg/kg 3N-3DU 10 hrs	5/8 (.56)	6.5 +/- 1.2 (.52)

3. JE virus target organ model

Work was conducted in an effort to establish a target organ model for evaluating anti-JE compounds. Preliminary titrations of JE virus Challenge Prep 8 were conducted to determine an intracranial LD_{90} dose. In the first titration, significant mortality only occurred at dilutions of 1:50,000 and lower when an inoculum volume of 0.03 ml was used. These results are shown in **Table 13.** A second titration was performed and the only inoculum dilution resulting in 100% mortality was 10^{-4}. At higher dilutions mortality occurred; however, it was not 100%. These results are shown in **Table 14.**

The effects of intracranial treatment with AVS 360 following intracranial JE Challenge were assessed. AVS 360 was administered intracranially at 3 mg/kg or 1 mg/kg in a volume of 0.03 ml at 6 or 8 or 10 hours post-JE inoculation. As shown in **Table 15,** 1 mg/kg of AVS 360 administered 10 hours post infection resulted in a mortality rate of 20% compared to 55.6% in the diluent treated virus infected control mice. These results indicate that a target organ system for testing antiviral compounds may be possible.

H. Conclusions

Studies conduted on this Contract provided several animals models which are applicable for assessing compounds with potential anti-encephalitis virus activity. Perhaps the models of greatest significance are the target organ systems whereby very small quantities of compounds can be assessed. No significant activity was found for any of the derivatives of ribavirin or selenazole which were tested.

Table 13

Preliminary Intracranial Titration of JE

Treatment Groups	No. Dead/Challenged	ADD ± 1 SD
Uninfected control	0/5	N/A
Sham-infected IC	0/10	N/A
1:10,000 dilution IC	10/10	8.0 ± 1.4
1:50,000 dilution IC	6/10	8.5 ± 2.8
1:80,000 dilution IC	4/10	9.3 ± 2.1
1:100,000 dilution IC	1/10	8.0 ± 0.0
1:200,000 dilution IC	2/10	11.5 ± 2.1
1:400,000 dilution IC	0/6	N/A
1:500,000 dilution IC	1/10	11.0 ± 0.0
1:600,000 dilution IC	0/10	N/A
1:800,000 dilution IC	0/10	N/A
1:1,000,000 dilution IC	0/10	N/A
1:2,000,000 dilution IC	0/10	N/A
1:4,000,000 dilution IC	0/10	N/A
1:6,000,000 dilution IC	0/10	N/A
1:8,000,000 dilution IC	0/10	N/A
1:10,000,000 dilution IC	0/10	N/A

Table 14

Secondary Intracranial JE Virus Challenge

Treatment Groups	No. Dead/Challenged	ADD ± 1 SD
Untreated control	0/5	N/A
Sham-infected IC	0/5	N/A
10^{-4} dilution JEV IC	10/10	8.4 ± 1.6
$10^{-4.3}$ dilution JEV IC	7/10	9.9 ± 3.4
$10^{-4.6}$ dilution JEV IC	6/10	10.2 ± 2.1
$10^{-4.9}$ dilution JEV IC	9/10	8.4 ± 1.0
$10^{-5.2}$ dilution JEV IC	3/10	9.7 ± 4.0
$10^{-5.5}$ dilution JEV IC	1/10	8.0 ± 0.0

Table 15

Results of Target Organ Treatment of JEV with AVS 360

Treatment	No. Dead/Uninfected	No. Dead/Infected	ADD ± 1 SD
6 hr post-infection			
HBSS		0/3	7/107.9 ± 0.9
3 mg/kg AVS 360	0/5	5/7	9.0 ± 2.3
1 mg/kg AVS 360	0/5	9/10	8.9 ± 1.6
8 hr post-infection			
HBSS		ND	9/108.1 ± 1.7
3 mg/kg AVS 360	ND	9/10	8.0 ± 1.1
1 mg/kg AVS 360	ND	8/10	8.3 ± 0.9
10 hr post-infection			
HBSS		ND	5/99.2 ± 2.3
3 mg/kg AVS 360	ND	6/10	8.0 ± 1.5
1 mg/kg AVS 360	ND	2/10	7.5 ± 0.7

ND = not done

V. LITERATURE CITED

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VI. ABSTRACTS AND PUBLICATIONS

No abstracts or publications were written on this project.

VII. LIST OF PERSONNEL RECEIVING CONTRACT SUPPORT, GRADUATE DEGREES RECEIVED

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