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DEVELOPMENT OF SYSTEMS FOR DELIVERY OF ANTIVIRAL DRUGS

ANNUAL REPORT

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This is our Second Annual Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period from September 30, 1986 through September 29, 1987.

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 are 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

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the parent drugs in the treatment of lethal Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus infections in mice. We synthesized the first of these prodrugs by a five-step route starting from ribavirin. In preliminary studies, this initial prodrug protected mice from a lethal challenge of JE virus and was much superior in efficacy to the parent drug, which had no effect. Extraction and HPLC assay procedures necessary for the proposed pharmacokinetic studies with the ribavirin prodrug in mice have been developed. Mice have been dosed with radiolabeled ribavirin prodrug and its metabolism and disposition has been determined with respect to time after i.v. administration. Little of the prodrug was detected in the brain, but the parent drug (ribavirin) was detected after 15 to 30 minutes. The prodrug was found to be rapidly eliminated from plasma (T1/2 <5 min.). The disappearance of the reduced prodrug corresponded with a progressive increase in the percentage of radioactivity present as the parent compound. Additional ribavirin prodrugs have been synthesized, some that are hydrolyzed more slowly than the original prodrug. Progress has also been made in the synthesis of the standard prodrug of selenazofurin. **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 are being repeated with the Beijing strain of JE virus administered i.p. to C57Bl/6 mice and using a chemoprophylaxis protocol as employed in the earlier studies at Fort Detrick. Additional **in vivo** studies with the other ribavirin prodrugs and the first selenazofurin prodrug are planned.

B

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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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 vitro** activity against selected DNA-containing and RNA-containinig 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 a prodrug form of these drugs would be desirable to develop.

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 gave 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 past year, we have concentrated on developing the 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. Useful information was obtained with regard to the importance of the virus strain, route of inoculation, and the test protocol for future antiviral evaluations in our laboratories at Southern Research Institute.

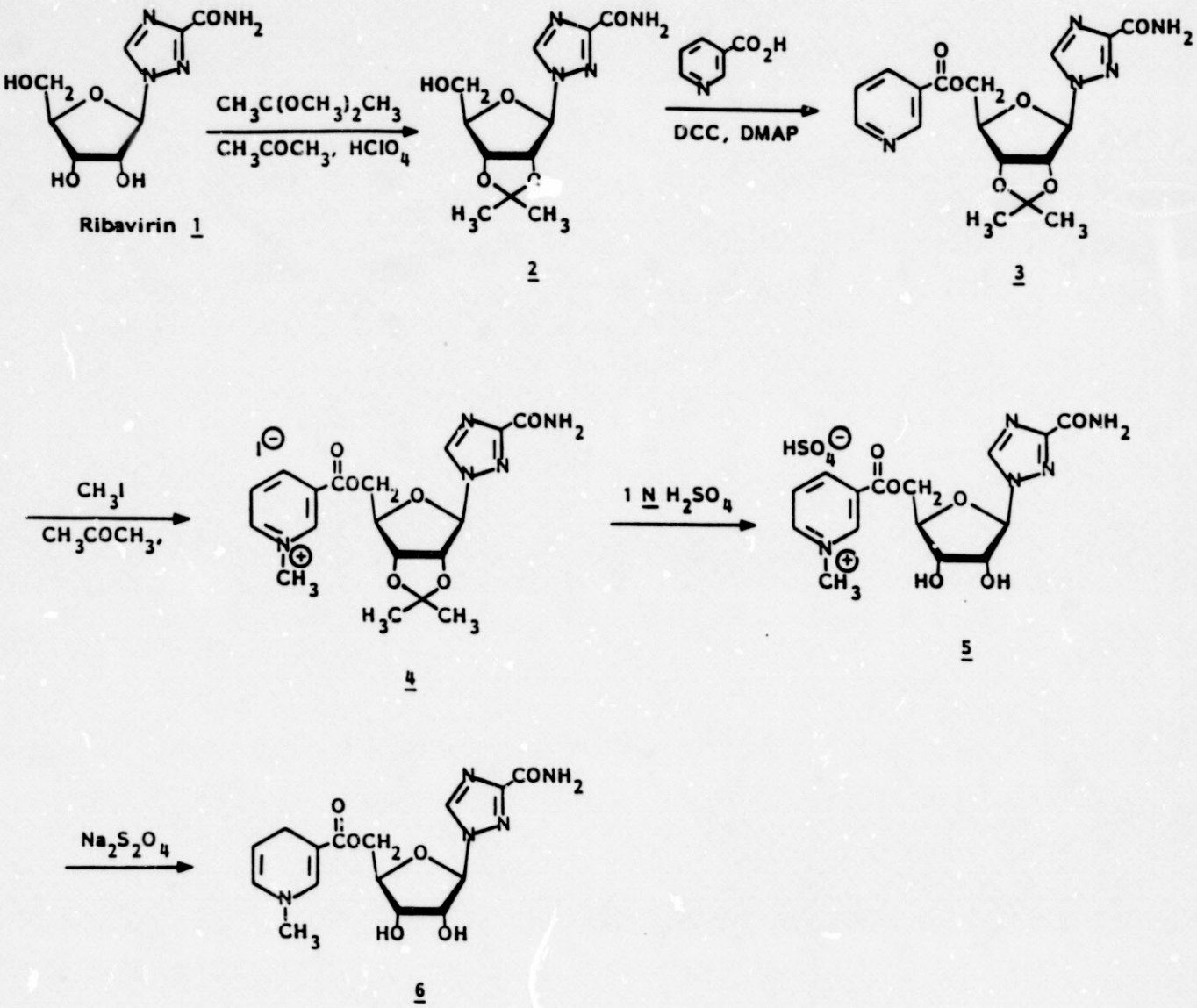
II. Chemistry

Our efforts during the past year have focused on the resynthesis of ribavirin prodrug **6** as well as the synthesis of several other prodrugs of ribavirin and one prodrug of selenazofurin. In addition, we have prepared ^{14}C-labelled **6**. During the latter part of the year, after we learned about the disposition of the radiolabelled **6**, we have begun to prepare ribavirin prodrugs that would be hydrolyzed more slowly than **6**.

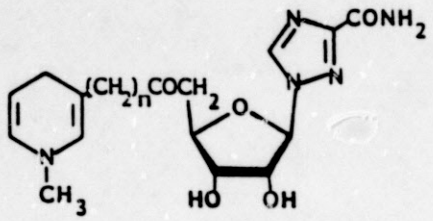
Scheme 1 shows how we have prepared **6** several times this past year. Initially, we had assumed that we probably would need to devise prodrugs that would be more easily hydrolyzed than **6**, and we set **7** and **8** as reasonable targets. Our proposed synthesis of **7** would go through **11** as shown in Scheme 2. 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 stage. Proper experimental conditions do allow the isolation of a tetrahydro compound. When the 2',3'-unblocked compound **11** was treated with sodium borohydride in anhydrous DMF, compounds **13-16** were formed. We were unsuccessful in our attempts to cleave the B-O bonds of compounds **15** and **16**, 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. Treatment of **17** 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 stage 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, which is waiting for animal testing, 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.

At the same time that we were pursuing **19** and related compounds, we were also making intermediates toward **8**. In that regard, we followed the route shown in Scheme 3 to arrive at **23**. As was the case with the lower homolog, reduction 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 **27** by the route shown in

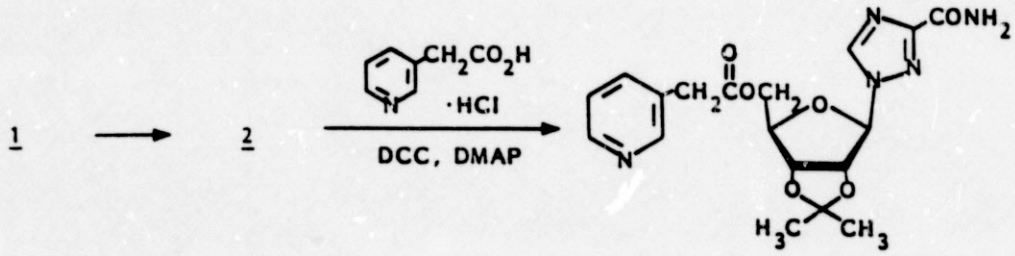
Scheme 1


Ribavirin 1
$CH_3C(OCH_3)_2CH_3$
CH_3COCH_3, $HClO_4$
2
DCC, DMAP
3
CH_3I
CH_3COCH_3,
4
1 N H_2SO_4
5
$Na_2S_2O_4$
6

Scheme 2



7: n = 1
8: n = 2



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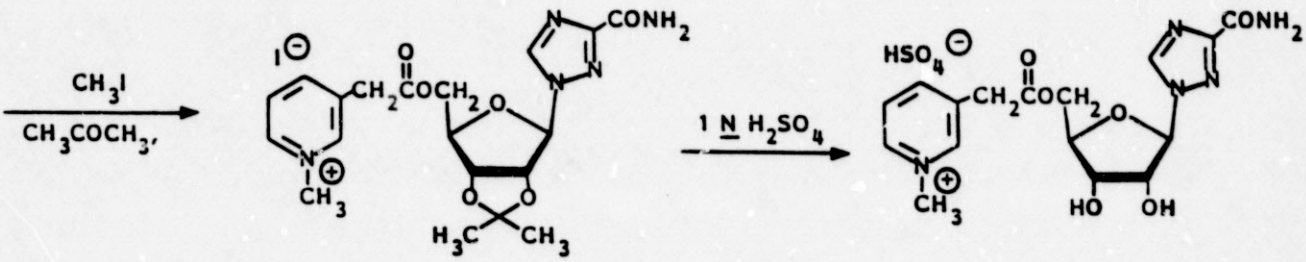

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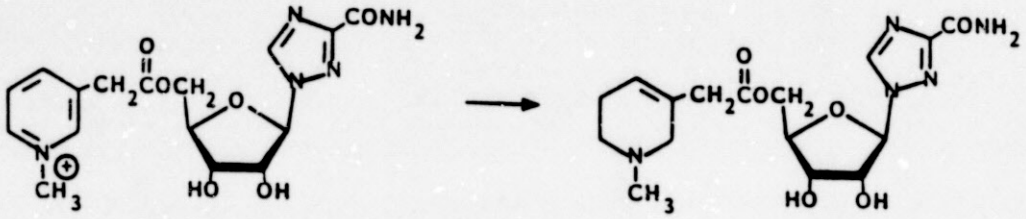
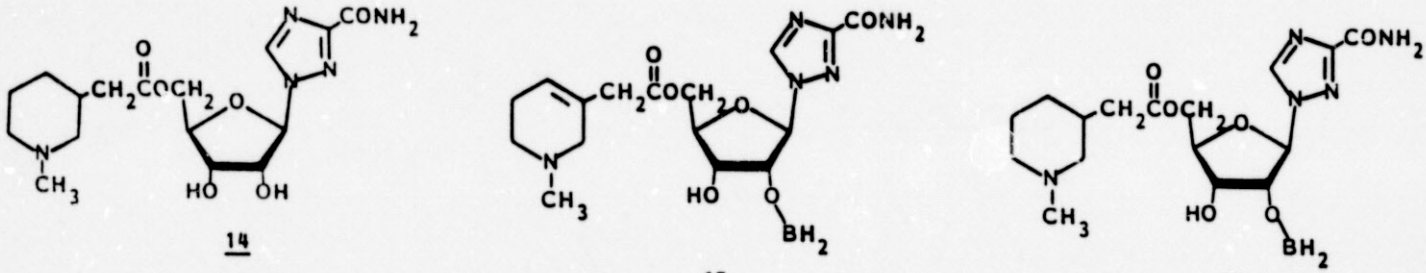
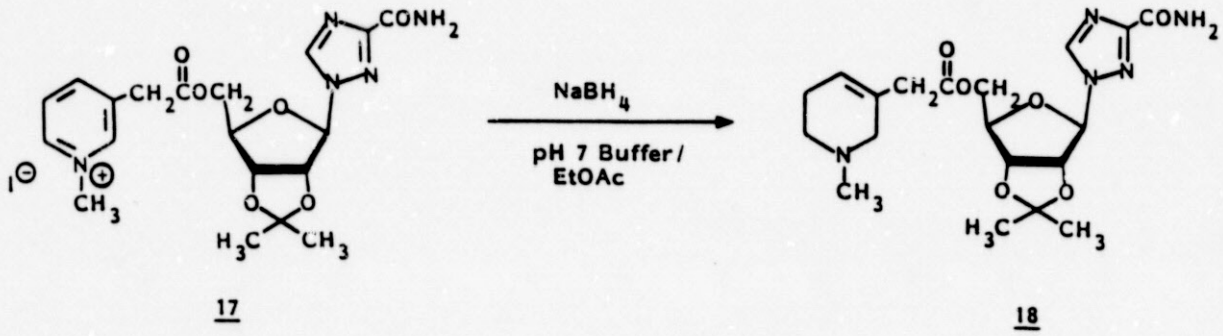


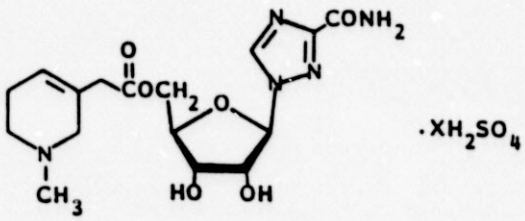
11 13



14 15 16

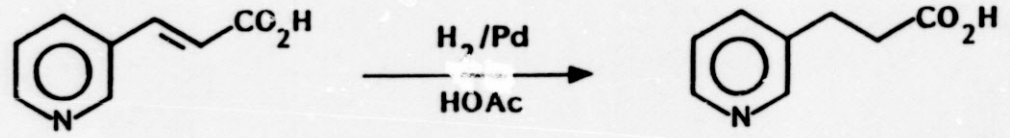


17 18



19

Scheme 3



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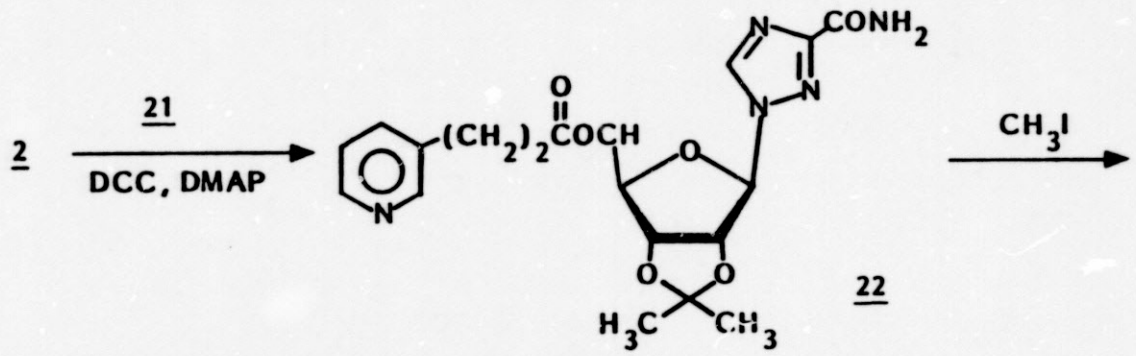

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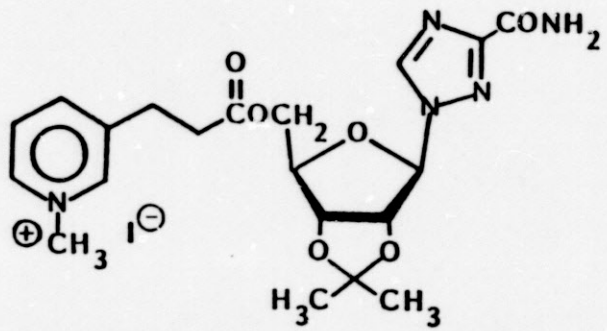


23

Scheme 4. Attempted reduction of **26** to **27** 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.

The synthesis of ^{14}C-labelled **6** 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 **6**, and these changes are noted in the Experimental Section. The synthesis provided ca. 9 mg of labelled **6** 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 **6**, the 1,2- and 1,6-dihydro isomers **28** and **29**. 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 **6**. Figure 1 shows that the prodrug is actually three isomers, the main one being the expected 1,4-dihydropyridine structure **6**, and the others presumably the 1,2- and 1,6-dihydropyridines **28** and **29**. 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 **6** 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 (**30**), we have prepared the standard prodrug **34** by the route shown in Scheme 5. 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. Because handling seems to reduce the purity of the compound, we plan to use what we prepare with a minimum of processing. We currently have 2.2 g of the precursor **33** on hand ready to use to prepare the prodrug.

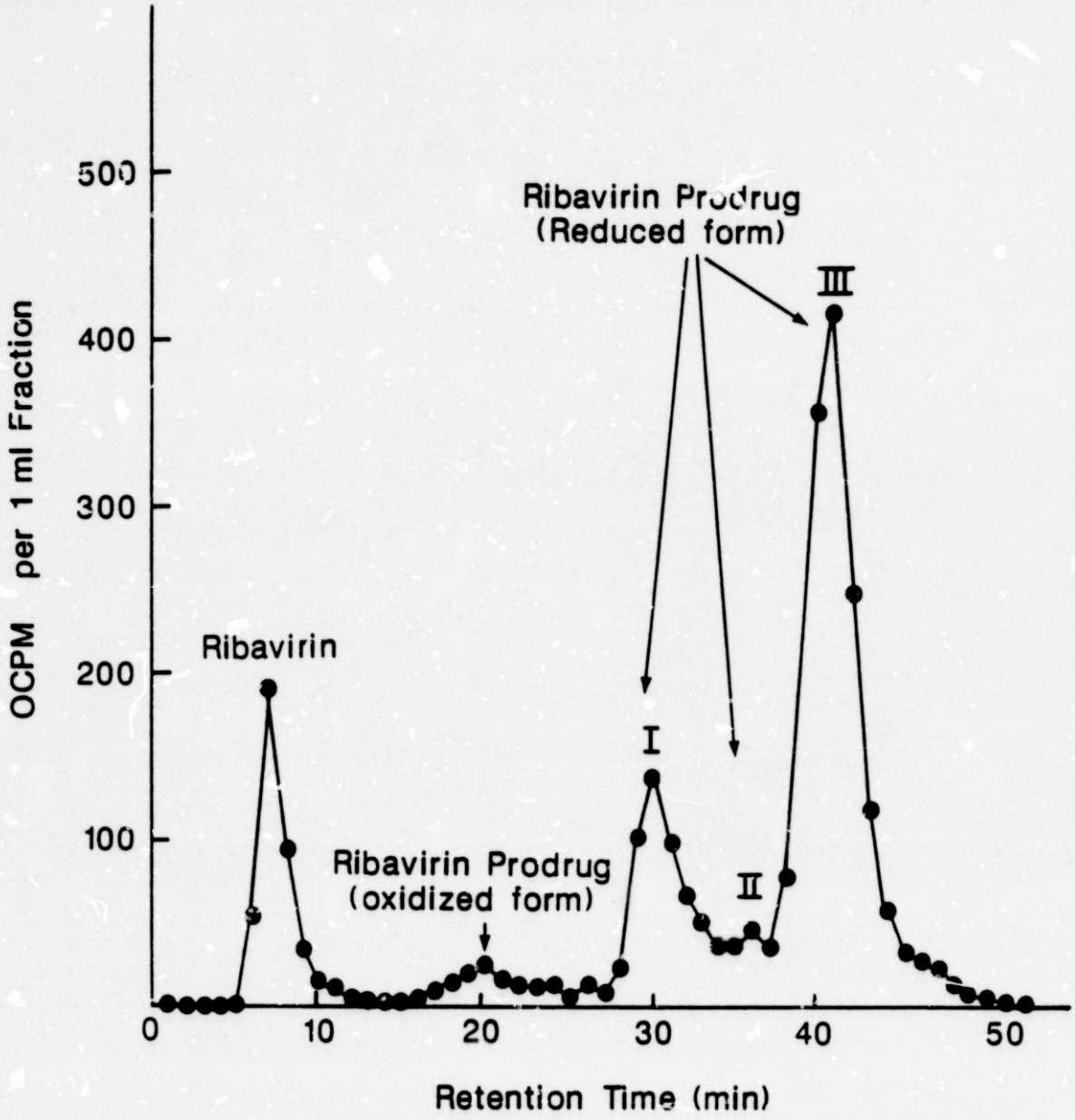


Figure 1. Analysis of [^{14}C] SRI 6711 by HPLC.
[^{14}C] SRI 6711 in H_2O was chromatographed as described in the text. One minute (1 ml) fractions were collected for radioassay. Radioassay data indicated that three radioactive compounds were present: ribavirin (16%) at approximately 8 min and two forms 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.

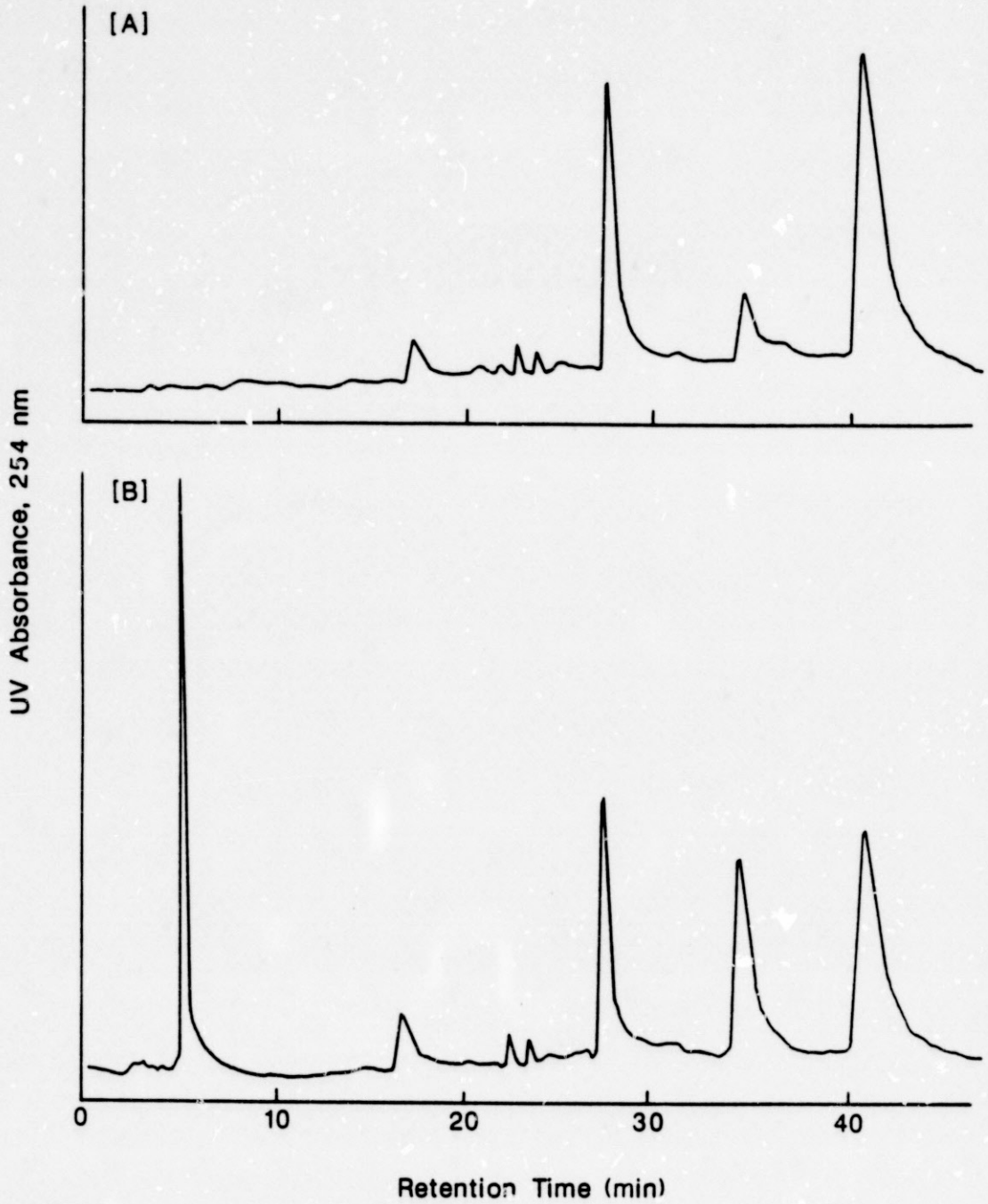


Figure 2. Stability of SRI 6711 in H_2O.
A freshly prepared aqueous solution of SRI 6711 was analyzed by HPLC, stored at -20° C for one week, and reanalyzed. While some breakdown to ribavirin did occur, the most significant change appeared to be the interconversion of the three forms of the reduced prodrug. 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.

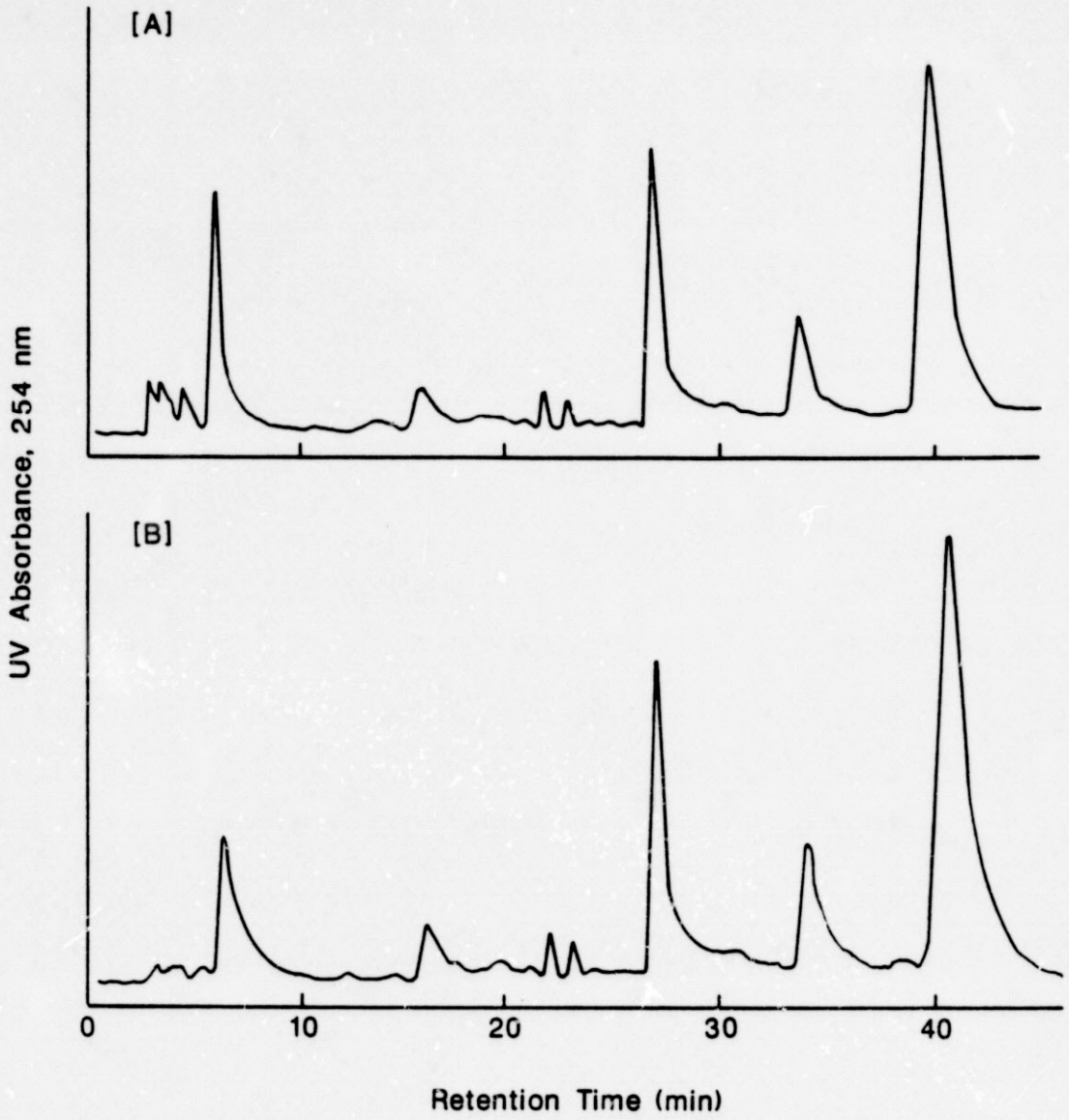


Figure 3. Effect of O_2 on an aqueous solution of SRI 6711.
A solution of SRI 6711 in water was treated with O_2 for 15 min. HPLC analyses of the solution before and after this treatment indicated that there were no significant changes in the composition of the sample as a result of this treatment. A,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

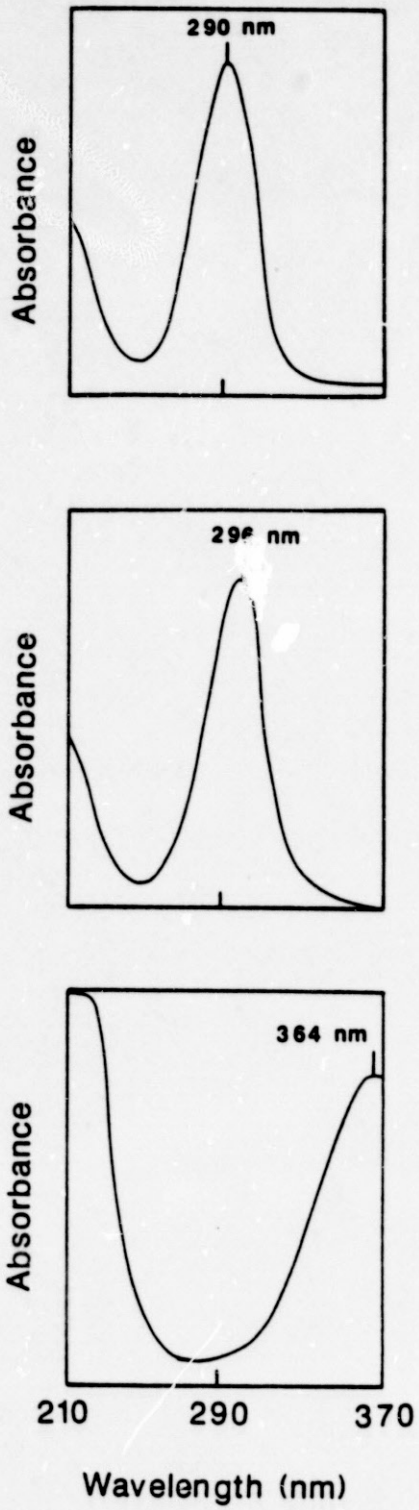
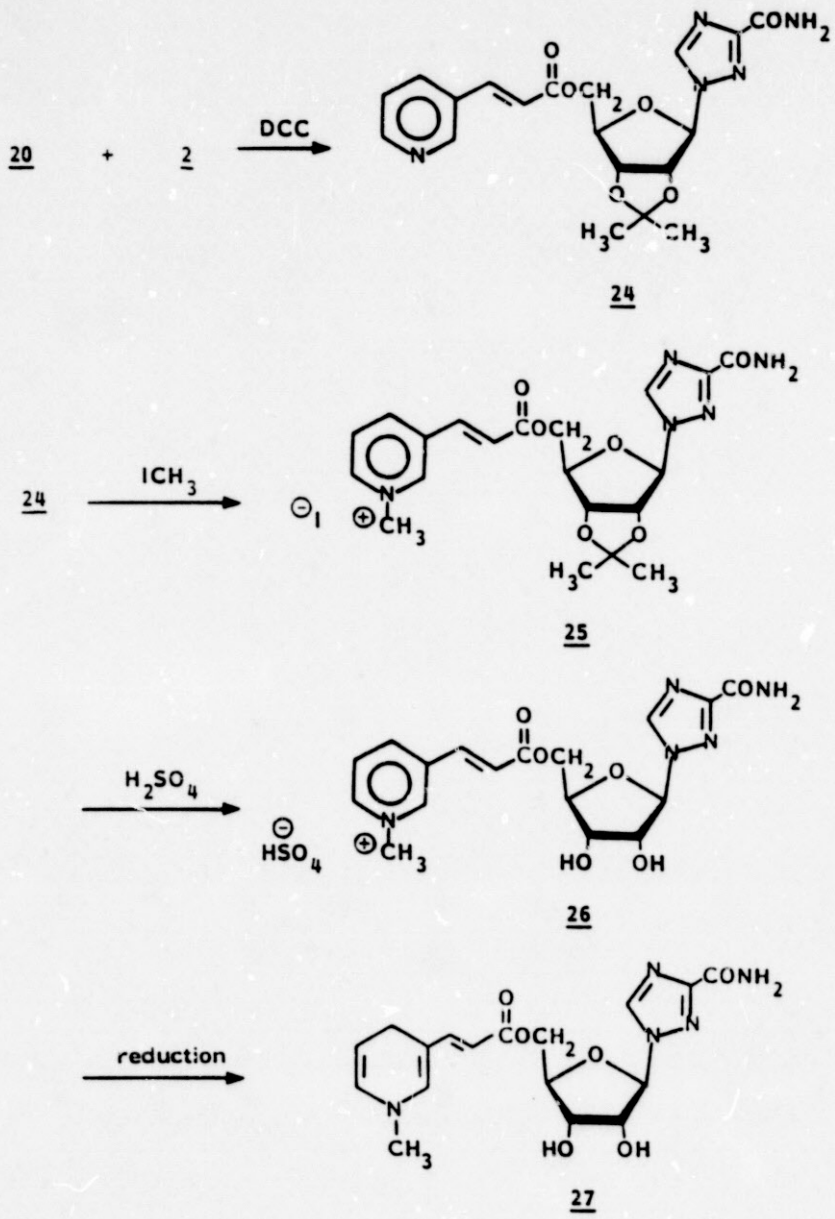
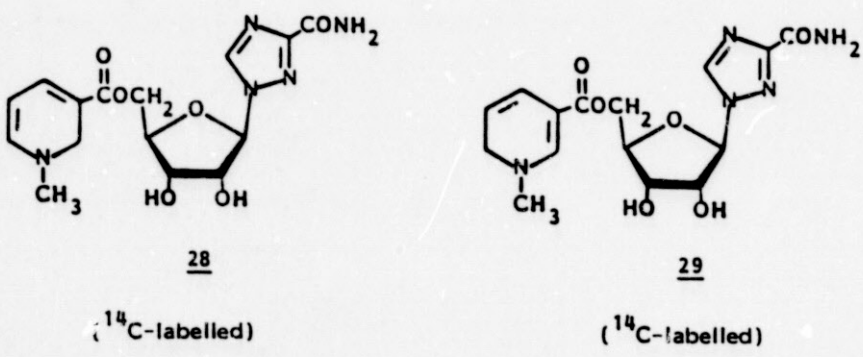


Figure 4. Ultraviolet absorption spectra of the peaks appearing between 25 and 40 min in the chromatograms shown in Figure 2B. The peaks eluting at 27, 34, and 40 min were scanned using an LKB 2140 scanning detector. A, peak eluting at 27 min with a UV max at 290 nm; B, peak eluting at 33 min with a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

Scheme 4

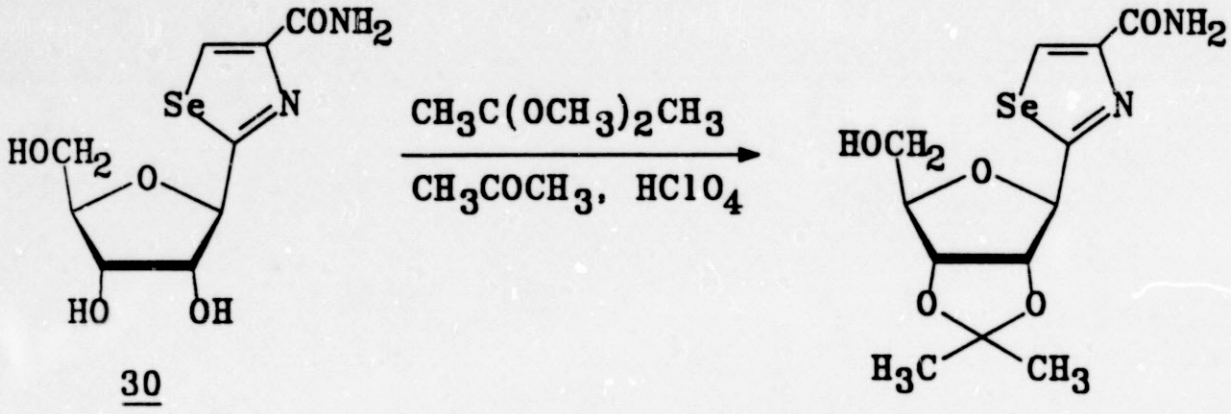




28 (14C-labelled)

29 (14C-labelled)

Scheme 5



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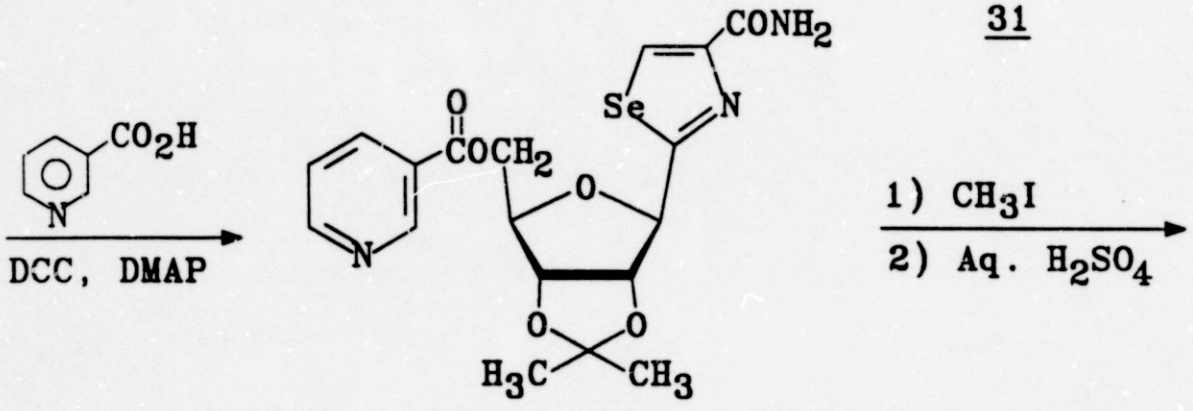

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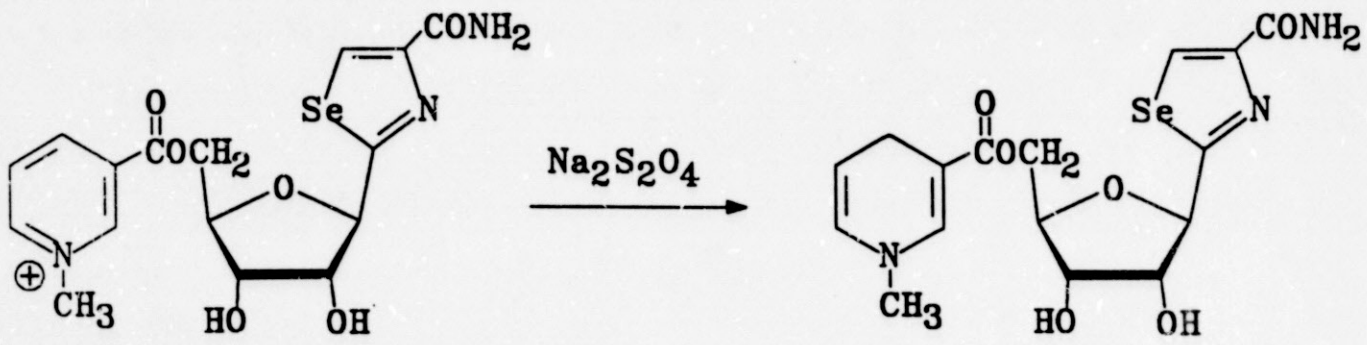
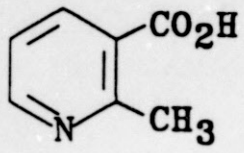


33 34



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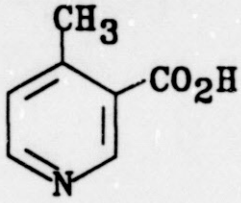

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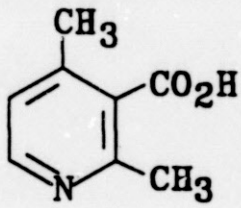

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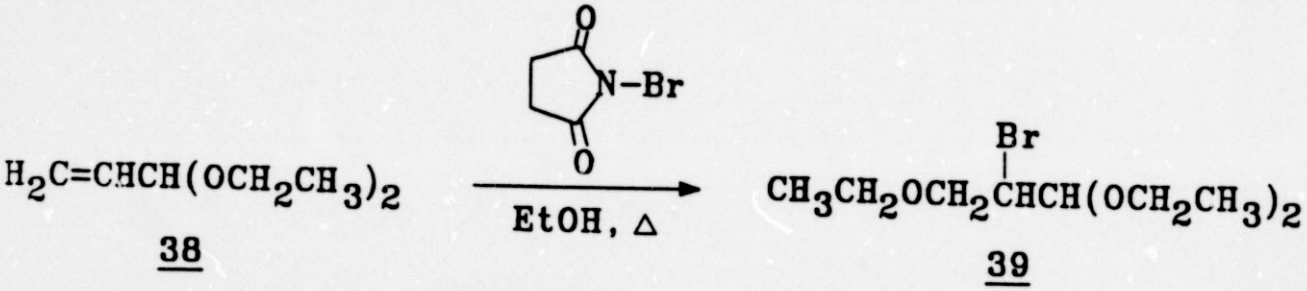
Scheme 6



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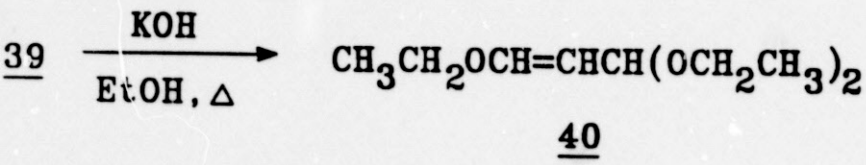

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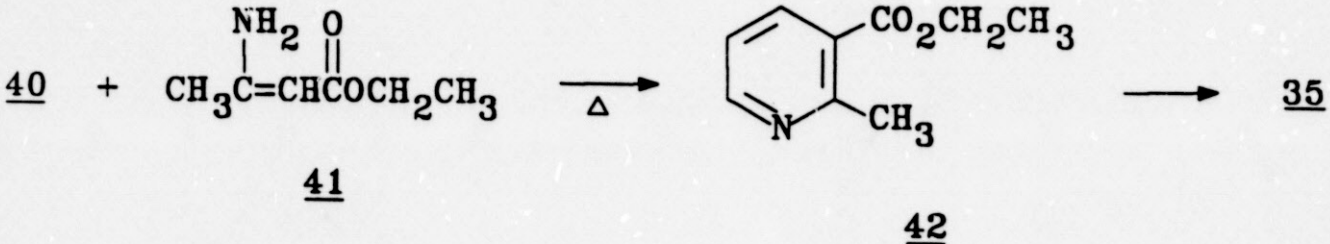
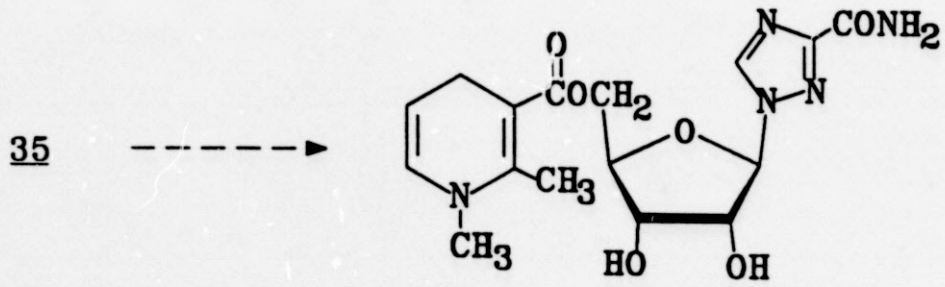


40 + 41 → 42 → 35



35 ------> 43

In embarking on a course that will produce prodrugs of ribavirin or other appropriate nucleoside antivirals that will be hydrolyzed more slowly than **6**, we first decided to keep the basic structure, that is, an ester of a nicotinic acid derivative as the 1,4-dihydropyridine. The most obvious way to slow down enzymatic (and chemical) hydrolysis is to increase steric bulk in the neighborhood of the ester carbonyl. The first step in such an approach logically would be to introduce methyl groups at the adjacent positions on the nicotinic acid. The synthesis of 2-methylnicotinic acid (**35**), 4-methylnicotinic acid (**36**), and 2,4-dimethylnicotinic acid (**37**), either as the free acids or their simple esters,[1-12] has been reported. We chose first to prepare **35** by the route shown in Scheme 6. Thus far we have prepared **40**, and we will soon be converting it on to the desired compound. Compound **35** will then be converted into prodrug **43**.

Future Plans

We will be preparing more of the initial prodrug **6** for comparison purposes with new prodrugs. We will submit the selenazofurin prodrug **34** for evaluation. At the same time, we will be developing new esters to be used in prodrugs that we hope will be more slowly hydrolyzed than **6**. The first target in this area is prodrug **43**, with a methyl group in a position that should interfere with ester hydrolysis. Directions following the preparation of **43** will depend upon how well it fits our desired criteria.

Experimental Section

2-(2,3-O-Methylethylidene-β-D-ribofuranosyl)selenazole-4-carboxamide (**31**). To a suspension of selenazofurin (**30**, 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 1N 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-5'a and H-5'b), 1.52 nd 1.32 (2 s, 6, $C(CH_3)_2$.

2-[2,3-O-Methylethylidene-5-O-(3-pyridinylcarbonyl)-β-D-ribofuranosyl]selenazole-4-carboxamide (**32**). A suspension of **31** (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-5'a), 4.45 (m, 1, H-5'b), 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 (33). A solution of 32 (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 1N 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-5'a), 4.54 (dd, 1, H-5'b), 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 (34). To a solution of 33 (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 (c/m 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. We presently have 2.2 g of the precursor 33 on hand.

1-[2,3-O-Methylethylidene-5-O-[3-(3-pyridinyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (24). To a mixture of 2',3'-O-methylethylideneribavirin (2, 1.50 g, 5.28 mmol), 3-(3-pyridyl)acryiic 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 DCU (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 (c/m 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 $(sugar)^+$. *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. ^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, -CH=CH-C(=O)-O], 7.45 (dd, 1, H-5"), 6.71 [d, 1, -CH=CH-C(=O)-O], 6.32 (s, 1, H-1'), 5.29 (d, 1, H-2'), 5.18 (dd, 1, -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, $C(CH_3)_2$).

1-[2,3-O-Methylethylidene-5-O-[3-(N-methyl-3-pyridiniumyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (25). Compound 24 (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; *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. ^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, -CH-CHCOO), 6.95 (d, 1, -CH=CHCOO), 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, $C(CH_3)_2$).

1-[5-O-[3-(N-methyl-3-pyridiniumyl)propenyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (26). Compound 25 (577 mg, 1.04 mmol) was stirred in 15 mL 1N H_2SO_4 at 40 °C for 1.5 h. The volume ws 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) 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,-CH=CHCOO), 7.02 (d, 1, -CH=CHCOO), 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, -CH=CHCOO), 133.70 (1, C-3"), 127.47 (1, C-5"), 124.21 (1, -CH=CHCOO-), 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.

1-[5-O-[N-methyl-[(3-pyridiniumylmethyl)carbonyl]]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (11). Compound 10 (1.72 g, 3.16 mmol) was stirred in 15 mL of 1N 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) β 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.

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);[1] EIMS 151 (M), 133 (M - H_2O)$^+$; ^1H NMR (Me_2SO-d_6) β 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_2CH_2COOH), 2.58 (t, 2, -CH_2CH_2COOH); CHN 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 (2, 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. ^1H 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, $-CH_2\underline{CH_2}COO-$), 2.58 (t, 2, $-\underline{CH_2}CH_2COO-$), 1.58 and 1.36 (2 s, 6, $C(CH_3)_2$).

1-[5-O-[N-Methyl-[(32-pyridiniumylethyl)carbonyl]]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (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%). ^1H NMR (Me_2SO-$\underline{d}_6$) 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, $-C=O-N\underline{H}_2$), 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-5'a), 4.10 (dd, 1, H-5'b), 3.00 (t, 2, $-C\underline{H}_2CH_2C=O-O-$), 2.75 (m, 2, $-CH_2C\underline{H}_2C=O-O-$), 1.51, 1.32 (2 s, 6, $C(CH_3)_2$). ^{13}C NMR (Me_2SO-$\underline{d}_6$) β 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 ($O_2\underline{C}(CH_3)_2$), 91.9 (C-1'), 85.0 (C-4'), 83.8 (C-2'), 81.1 (C-3'), 63.9 (C-5'), 47.7 ($N^+-\underline{C}H_3$), 32.8 ($-CH_2C\underline{H}_2C=O-O-$), 26.6 ($-\underline{C}H_2CH_2C=O-O-$), 26.6, 24.9 ($\underline{C}(CH_3)_2$). M.p. 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-β-(2,3-O-Methylethylidene-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide-^{14}C (2). 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 μl of 2.2 dimethoxypropane followed by 2.7 μl 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 (9:1 $CHCl_3$-MeOH, 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]-1H-1,2,4-triazole-3-carboxamide-^{14}C (3). A solution of 2 (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]-1H-1,2,4-triazole-3-carboxamide-^{14}C Iodide (4). A solution of 3 (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]-1H-1,2,4-triazole-3-carboxamide-^{14}C Sulfate Salt (5). A solution of 20 mg (0.051 mmol) of 4 in 1N 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%). The material was stored at -20 °C under argon until used in a reaction.

1-[5-O-(N-Methyl-1,4-dihydropyridinylcarbonyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide-^{14}C (6). A solution of 5 (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 μl 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.

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III. PHARMACOLOGY AND HPLC ASSAY PROCEDURES

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 anesthesized 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 (Fig. 1). Elution at final conditions was continued for 20 min. The column was equilibrated at initial conditions for 30 min between analyses. One minute fractions of 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 less than 5 min (Figure 2). 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 greater than 9 hr (Figure 2). 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 1 and 3 and Tables 1 and 2. Little radioactivity (less than 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 3). 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 $\pm$ 3) of the administered dose was excreted into urine within 24 hr. Of the radioactivity recovered, 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 [^{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 (less than 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.

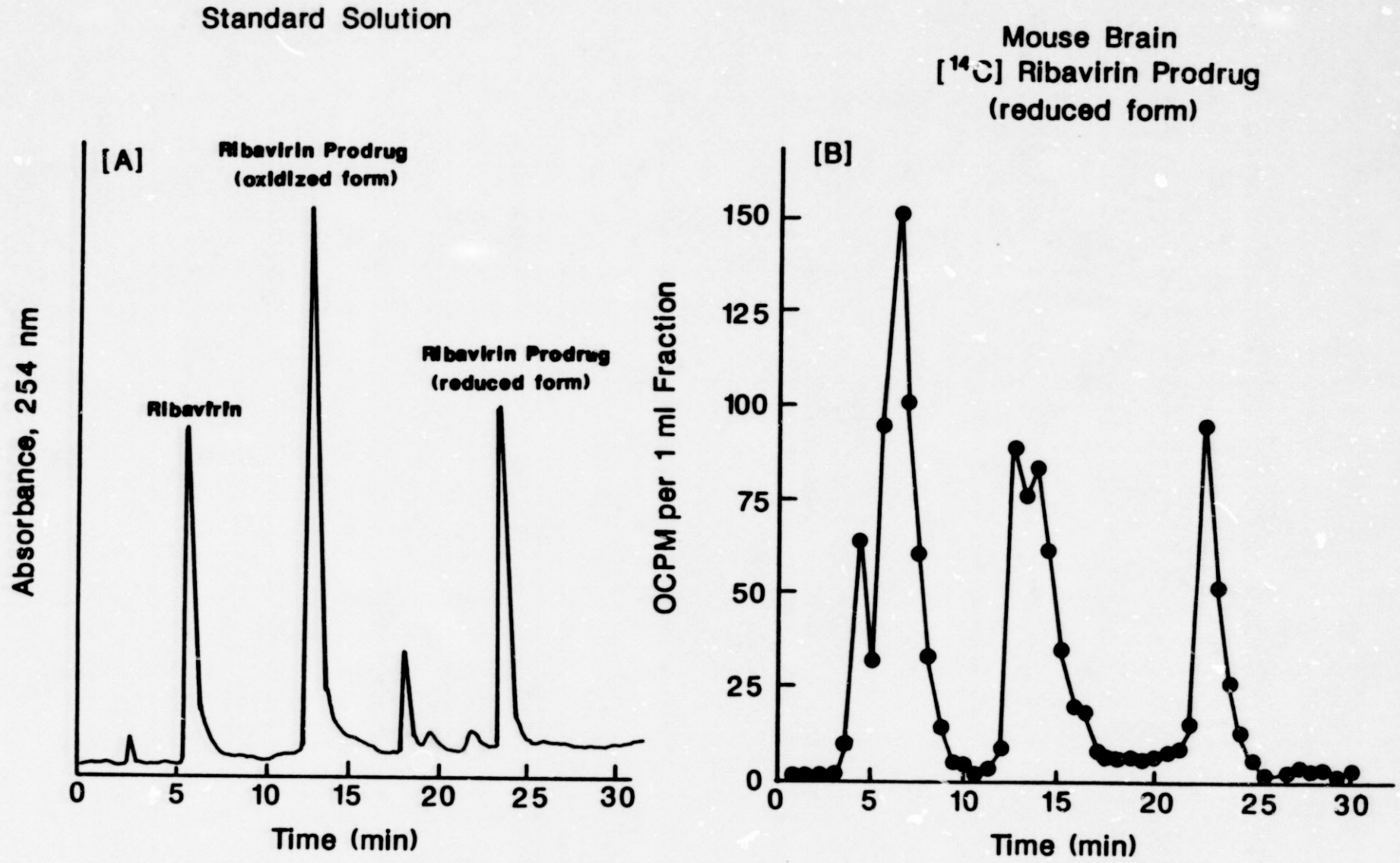

Figure 1. HPLC analyses of [A] a standard solution containing the reduced and oxidized forms of SRI 6711 (ribavirin prodrug) and ribavirin; [B] an extract of mouse brain obtained 30 min after administration of 50 mg/kg of [^{14}C] SRI-6711.

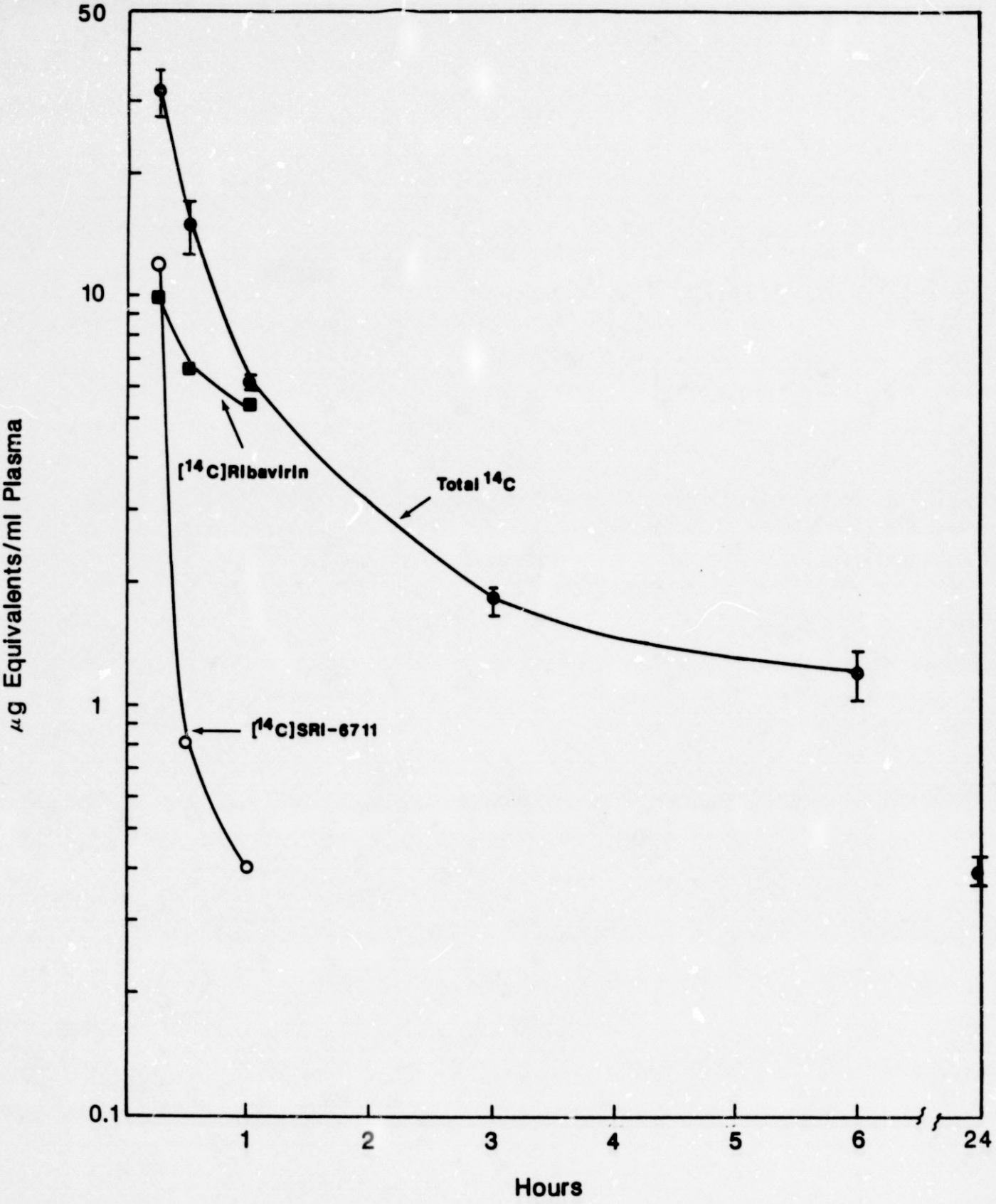

Figure 2. Plasma elimination of total radioactivity (●), [^{14}C] SRI-6711 (O) and [^{14}C] ribavirin (■) following i.v. administration of [^{14}C] SRI-6711 to mice.

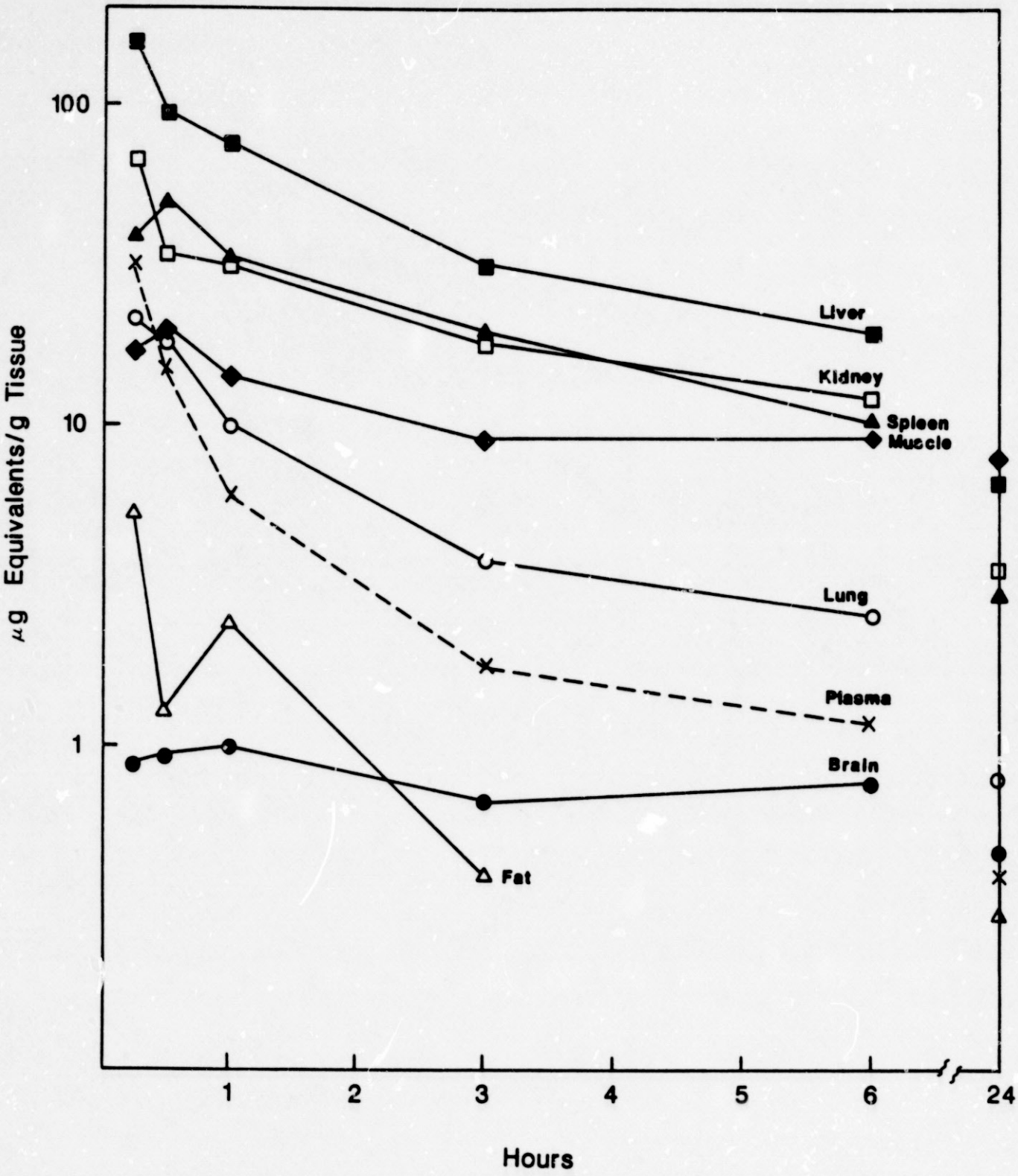

Figure 3. Tissue levels of total radioactivity following i.v. administration of [^{14}C] SRI-6711 to mice.

Table 1

HPLC Analyses of Plasma, Urine and Tissue Extracts
Obtained from Mice Administered [^{14}C] SRI-6711

Tissue	Time	Mouse No.	µg equiv. per 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 Administration of [^{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

IV. VIROLOGY

A. Personnel Immunization Program

Since the inception of the immunization program in February, 1986, sixty people have completed the program. Sixteen of these people completed the program during the current reporting period. Of these 16, 12 (75%) seroconverted to antibody titers of 20 or greater against VEE after receiving the live, attenuated vaccine (TC-83). Three of the remaining 4 (25%) people received the inactivated VEE booster immunization and all 3 (100%) seroconverted. In comparison, 68% of the previous year's vaccinees seroconverted to VEE after receiving the live vaccine and only 75% of those given the inactivated booster seroconverted.

In regards to the JE vaccinations, eight (50%) of the 16 new vaccines seroconverted to antibody titers of 10 or greater against JE after receiving the initial three doses of inactivated vaccine. This is compared to a 60% seroconversion rate the previous year. None of the remaining 8 (50%) people were given a booster immunization because these people would not be working directly with the virus and supplies of the vaccine are severely limited.

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 during the last contract year. 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 are produced in Vero cell cultures. Each working stock is titrated by plaque and end-point CPE assays in Vero cell cultures. Working stocks of each virus are 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 has been titrated and frozen for use in the **in vivo** challenge studies.

C. **In Vivo** Titration of 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 initraperitoneal (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. **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 free (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 (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 indicate that the LD_{90} for second passage JE virus administered by the IC route is approximately 1.1 pfu/mouse.

3. Enhancement of JE Virulence for IP Inoculation

In an attempt to generate a pool of the Nakayama strain of JE virus which is 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.

4. **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 to date with this strain of virus following IP challenge in CD-1 mice is extremely variable and does not provide a viable model. Serial **in vivo** passage of this virus is currently in progress in an effort to enhance the intraperitoneal pathogenicity. The virus will then be titrated in C57B1/6 mice which are more sensitive to its lethal effects.

5. **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_{50} for the VEE virus administered IC is 0.051 pfu/mouse while the LD_{50} for IP administered virus is 0.45 pfu/mouse. The LD_{90} VEE virus 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 ranges from 7.6 days to 11.8 days. The ADD for IC challenged animals ranges from 6.2 days to 8.5 days. The LD_{50} is less than 1 pfu/mouse which may be due to differences in **in vitro** and **in vivo** virulence. A similar effect was reported by Taylor et al. (1980. J. Infect. Dis. 142:394-399) in JE virus challenge studies performed in mice.

D. **In Vivo** Evaluations of the Ribavirin Prodrug for Antiviral Activity

1. 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 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 1, 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.

2. 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 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 2, 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. Greater efficacy might have occurred if a prophylactic treatment schedule and intraperitoneal virus challenge had been employed as was performed at Fort Detrick. Additionally, the antiviral assessment of this compound against JE virus infection performed at Fort Detrick utilized the Beijing strain of JE virus while the work at SRI was conducted with the Nakayama strain of JE virus. The use of the Beijing strain of JE virus allows one to obtain reproducible virus-induced mortality after intraperitoneal virus challenge. We therefore believe that this strain is preferable for antiviral chemotherapy studies. Intracranial challenge results in a fulminant infection which may be too severe for the assessment of antiviral prodrug efficacy.

Table 1

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

Treatment Group	Drug Dose mg/kg	Day 21 Mortality		
		Uninfected	Infected	
		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.

Table 2

Results of Prodrug Activity in JE Virus Infected Mice

Treatment[a]	No. Dead/ No. Uninfected	No. Dead/ 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.

E. Future Plans

Virology

During the next year we will continue our work aimed at developing a viable JE virus model for use in intraperitoneal challenge studies. We have obtained the Beijing strain of JE virus from Fort Detrick for this purpose.

We anticipate that **in vivo** antiviral compound evaluations will continue during the next year. For these studies we will use a prophylactic rather than a therapeutic protocol during the initial **in vivo** evaluation in order to maximize the potential antiviral efficacy of the test compounds.

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