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THE ANTI-VIRAL ACTION OF A RIFAMYCIN DERIVATIVE: FORMATION OF ROUS SARCOMA VIRUS PARTICLES DEFICIENT IN 60-70S RNA

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The Anti-Viral Action of a Rifamycin Derivative: Formation of Rous Sarcoma Virus Particles Deficient in 60-70S RNA

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The antibiotic Rifamycin SV and its derivatives show inhibitory activity in a wide range of biological systems. Certain derivatives act as anti-bacterial agents by specifically inhibiting the action of bacterial DNA-dependent RNA polymerase[1-3]. In addition, one of these compounds rifampicin (rifampin) inhibits pox virus replication in cell culture by a different mechanism[4-6]. Many derivatives have been screened for selective inhibition of viral RNA-dependent DNA polymerase (reverse transcriptase) and certain of these derivatives prevent focus formation by RNA tumor viruses in cell culture[7-8].

One derivative, Rifazone-8_2 (R-8_2), synthesized in this laboratory[9] is a potent inhibitor of viral reverse transcriptase and possesses a number of other interesting properties. R-8_2 preferentially inhibits the growth of virus-transformed cells in culture[10]. Recently, R-8_2 has been shown to have anti-viral activity as well; the drug prevents the spread of Rous sarcoma virus (RSV) infection when added to cultures in which only a fraction of the cells are infected[11]. The replication of the virus is not inhibited as measured by the production of physical particles but the drug causes the formation of non-infectious particles.

In this communication the mechanism of anti-viral activity is examined in detail. Growth of RSV transformed cells in the presence of R-8_2 resulted in the synthesis of non-infectious virions with an altered buoyant density. Studies with ^{3}H-labeled R-8_2 showed that the drug was bound to the virus even after extensive purification. Analysis of the RNA extracted from the non-infectious virus indicated that most of the RNA (>95%) appeared as small species and only a minor fraction of 60-70S RNA was detected.

The ability of R-8_2 to cause the production of non-infectious virus has been described[11]. Addition of 15 µg/ml of R-8_2 greatly reduced infectious

particle synthesis while the production of physical virus particles was only slightly affected. The concentration dependence of this inhibition was tested by growing Prague-C (PR-C) transformed cells in various concentrations of R-8_2. The addition of 5 μg/ml resulted in a slight decrease in the synthesis of physical particles as measured by reverse transcriptase activity, and caused a 60% reduction in focus-forming activity of virus after 2 days of treatment (Fig. 1). Addition of 15 μg/ml of R-8_2 reduced the infectivity of the progeny virus by greater than 99% after only 1 day while particle production was reduced much more slowly. These results indicate that the virus infectivity is much more sensitive to inhibition by R-8_2 than is virus reproduction.

Fig. 1 also shows the effect of rifampicin on RSV reproduction and infectivity. Growth of the transformed cells in 15 μg/ml of rifampicin had negligible effect on both virus particle production and virus infectivity. This suggests that the anti-viral activity of R-8_2 is due, in part, to the lipophilic side chain of the compound since R-8_2 and rifampicin share the same ansa ring backbone[12].

Most of the original anti-viral studies were performed with the Prague-C strain of RSV. Cells infected with the Bryan or Schmidt-Ruppin-A (SR-A) strain of RSV and grown in the presence of 15 μg/ml of R-8_2 gave similar results. After 24 h of treatment the infectivity of the progeny virus was only 5 to 10% of that of the untreated virus and after 2 days the focus forming ability of the progeny virus was reduced 10^{-3}-fold (results not shown).

Non-infectious virus released from R-8_2 treated cells was labeled with ^{3}H-uridine and analyzed by sucrose equilibrium sedimentation (Fig. 2).

RSV virions normally sediment in a sharp band with a median density of 1.16 g/cc. Analysis of drug-treated virus revealed a broadening in the peak and a decrease in the buoyant density with the activity centered at 1.14 g/cc. The viral reverse transcriptase activity co-sedimented with the labeled virus band. The change in the buoyant density profile was characteristic of all RSV preparations grown in the presence of the drug. However, the infectivity of the treated virus was at least 200-fold lower in all fractions across the gradient, even those with a density of 1.16 g/cc (not shown). Thus, growth in R-8_2 leads to a loss of infectivity irrespective of the density of the virus.

Since R-8_2 is a highly lipophilic compound, one possible explanation of the change in density of the R-8_2 treated virus is that the drug, through its association with the viral envelope, disrupts the normal compact structure of the virion. Studies with ^{3}H-labeled R-8_2, synthesized in this laboratory, indicated that appreciable amounts of the drug remained associated with the virus even after purification through two sucrose gradients (Fig. 3). PR-C was grown in the presence of 15 µg/ml of ^{3}H-R-8_2 for 24 h, collected by centrifugation, washed and banded in a sucrose gradient (Panel A). The peak fractions were pooled, concentrated, and centrifuged on a second gradient (C). The peak of reverse transcriptase activity, representing virus particles, cosedimented with the ^{3}H-R-8_2 peak at a density of 1.14 g/cc and the two could not be separated by repeated washing and centrifugation. The infectivity of the virus band was 100-fold lower than control virus. Mature RSV, incubated for 1 h at 37° in medium containing ^{3}H-R-8_2, was treated as described above (Panels B and D). Although

detectable amounts of R-8_2 were bound during the preincubation, the virus buoyant density and the infectivity of the virus were unaffected. Thus a simple binding of the drug to mature virus is not sufficient to cause a change in buoyant density or infectivity.

Hunter et al.[14] have described a RSV mutant that is temperature sensitive for virus assembly. When grown at the non-permissive temperature the progeny virus is non-infectious, has an altered buoyant density, and contains an excess of 4S RNA over 70S RNA. To determine if the binding of the drug and the change in buoyant density has any effect on the viral RNA, ^{3}H-uridine labeled RNA from R-8_2 treated and untreated virus was phenol extracted and subjected to velocity sedimentation in glycerol (Fig. 4). RNA from untreated virus yielded a fast-sedimenting peak of activity at approximately 70S and some smaller RNA at the top of the gradient (A). However, nearly all the RNA from the drug treated virus was found at the top of the gradient and only a small amount of 60-70S RNA was detectable (B).

Treatment of mature virus with 15 µg/ml of R-8_2 before phenol extraction did not affect the sedimentation profile of the RNA (C). Thus the great excess of small RNA was detected only in the non-infectious virus grown in the presence of R-8_2.

The lack of 60-70S RNA in the non-infectious virions appears to be the ultimate cause for loss of infectivity. The drug itself does not appear to affect the integrity of the RNA. Treatment of mature virions with 15 µg/ml of R-8_2 does not alter the proportion of 60-70S to 4S RNA.

Evidence presented here suggests that interaction of the drug with the virus during maturation and release could be responsible for the anti-viral activity of R-8_2. An estimated 10^3 to 10^4 molecules of R-8_2 per virion are associated with virus grown in the presence of the drug and the compound cannot be removed by repeated washing. The buoyant density of the virus is altered from a characteristic sharp peak at 1.16 g/cc in sucrose

gradients to a broad band centered at 1.14 g/cc. Incubation of R-8_2 with mature virions does not alter the buoyant density of the virus or significantly lower the infectivity although some R-8_2 cosediments with the virus peak.

It is possible that R-8_2 associates with the virus during budding and disrupts the normal compact structure of the viron. This may prevent the stabilization of the viral 60-70S RNA[15,16] and ultimately lead to its degradation. Alternatively, acting as nucleotide polymerase inhibitor, R-8_2 may prevent the synthesis of the 30-40S subunits of the viral RNA which results in the packaging of the small RNA. The origin and the nature of the small RNA is under investigation.

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Figure Legends:

Fig. 1. Effect of R-8_2 and rifampicin on RSV reproduction and infectivity. Chick embryo fibroblasts in medium 199 were infected with PR-C RSV at the time of primary seeding[10]. Secondary cultures were prepared after 4 days at a density of 2 x 10^6 cells per 60 mm dish and treated with either R-8_2 or rifampicin as indicated. The supernatant fluids were collected every 24 h and replaced with fresh media containing the appropriate drug. Virus particles were collected by centrifugation, washed in standard buffer (0.01M Tris pH 7.4,0.1M NaCl, 0.001M EDTA) and assayed for viral reverse transcriptase activity with the addition of poly(rC): oligo (dG) (open bars) and focus-forming activity (hatched bars)[11]. Data are presented relative to control values after 1 day of treatment (A) and 2 days of treatment (B).

Fig. 2 Equilibrium density analysis of RSV grown in the presence of 15 µg/ml of R-8_2. PR-C infected cultures were grown as described for Fig. 1. After 1 day of drug treatment ^{3}H-uridine (10 µCi/ml) was added to the cultures for 8 h. ^{3}H-labeled virus from the culture fluids of control (A) and R-8_2 treated (B) cultures were collected by centrifugation, resuspended in standard buffer and subjected to equilibrium density centrifugation (3 h at 45,000 RPM in the Spinco SW 50.1 rotor) in 25-60% sucrose gradients. Aliquots (100 µl) from each fraction were treated with 3 ml of 5% trichloracetic acid (TCA), acid insoluble material was collected

on Millpore filters and the ^{3}H-uridine (O) was measured by liquid scintillation counting. A 10 µl aliquot from each function was assayed for viral reverse transcriptase activity (●) as described for Fig. 1.

Fig. 3. Equilibrium sedimentation of RSV treated with ^{3}H-labeled R-8$_2$. A PR-C infected culture was grown in the presence of 15 µg/ml of ^{3}H-labeled R-8$_2$ (100 µCi/µmole) for 24 h. Virus was collected by centrifugation, washed and banded in sucrose as described for Fig. 2 (A). The virus in the peak fractions was collected by centrifugation, resuspended in standard buffer and sedimented on a second gradient (C). Mature RSV from untreated control cultures was incubated in medium containing ^{3}H-R-8$_2$ for 1 hr at 37° and then treated as described above (B and D). Each fraction was assayed for viral reverse transcriptase activity[11] (O) and for TCA insoluble ^{3}H-R-8$_2$ (●).

Fig. 4. Sedimentation analysis of viral RNA. Infected cultures were grown and labeled with ^{3}H-uridine and virus was purified by equilibrium sedimentation in sucrose as described for Fig. 3. Viral RNA from (A) untreated control cultures and (B) cultures grown in the presence of 15 µg/ml of R-8$_2$ was extracted by the phenol-sodium dodecyl sulfate method[15]. The viral RNA was then subjected to

to velocity sedimentation for 75 min at 45,000 RPM in the Spinco SW 50.1 rotor in a 15 to 30% glycerol gradient. ^{3}H-labeled RNA was measured by taking aliquots of each gradient fraction into Aquasol. In (C), mature virus from untreated control cultures was incubated in medium containing 15 μg/ml of R-8_2 for 1 hr at 37°. The RNA was extracted and analyzed as described above.

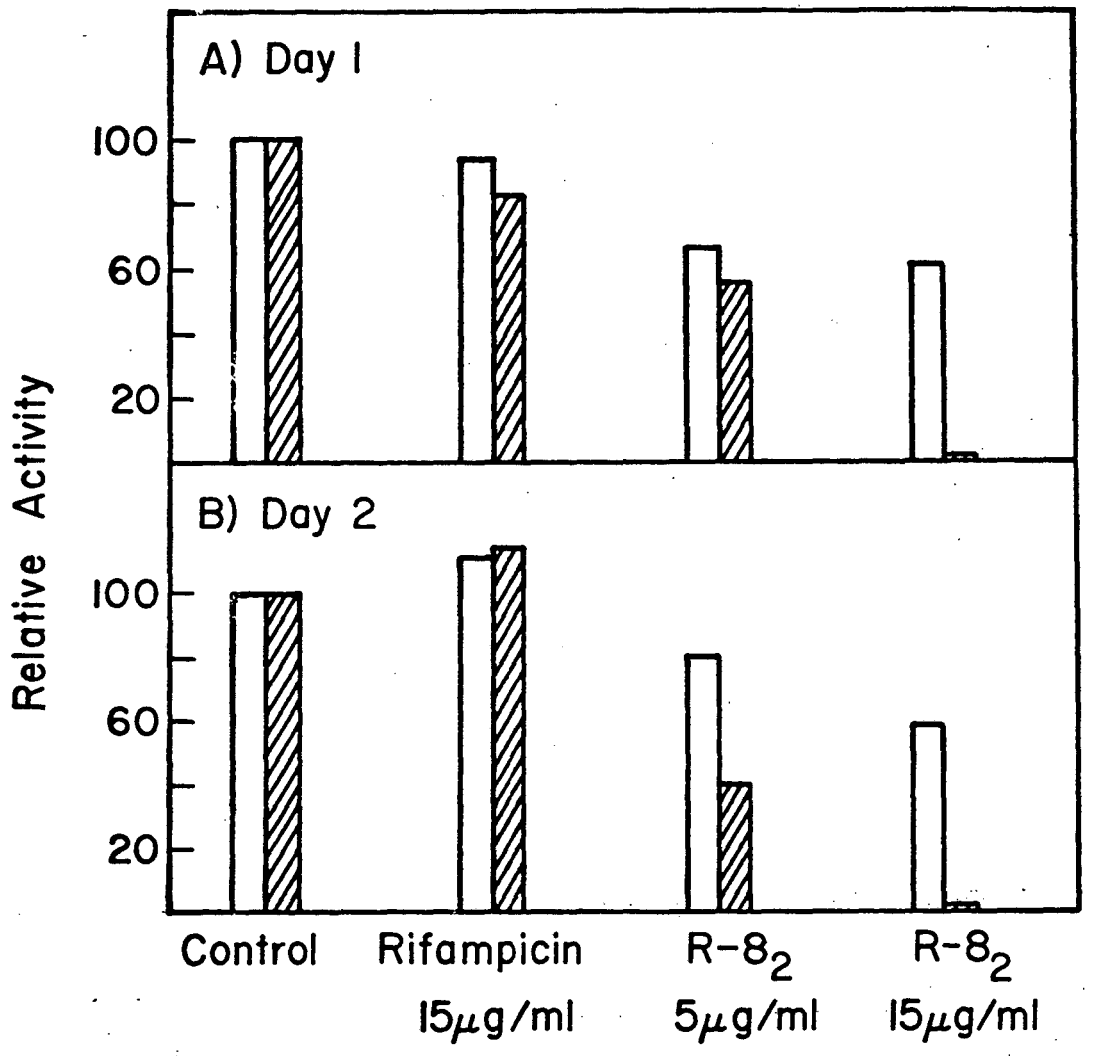

Figure 1

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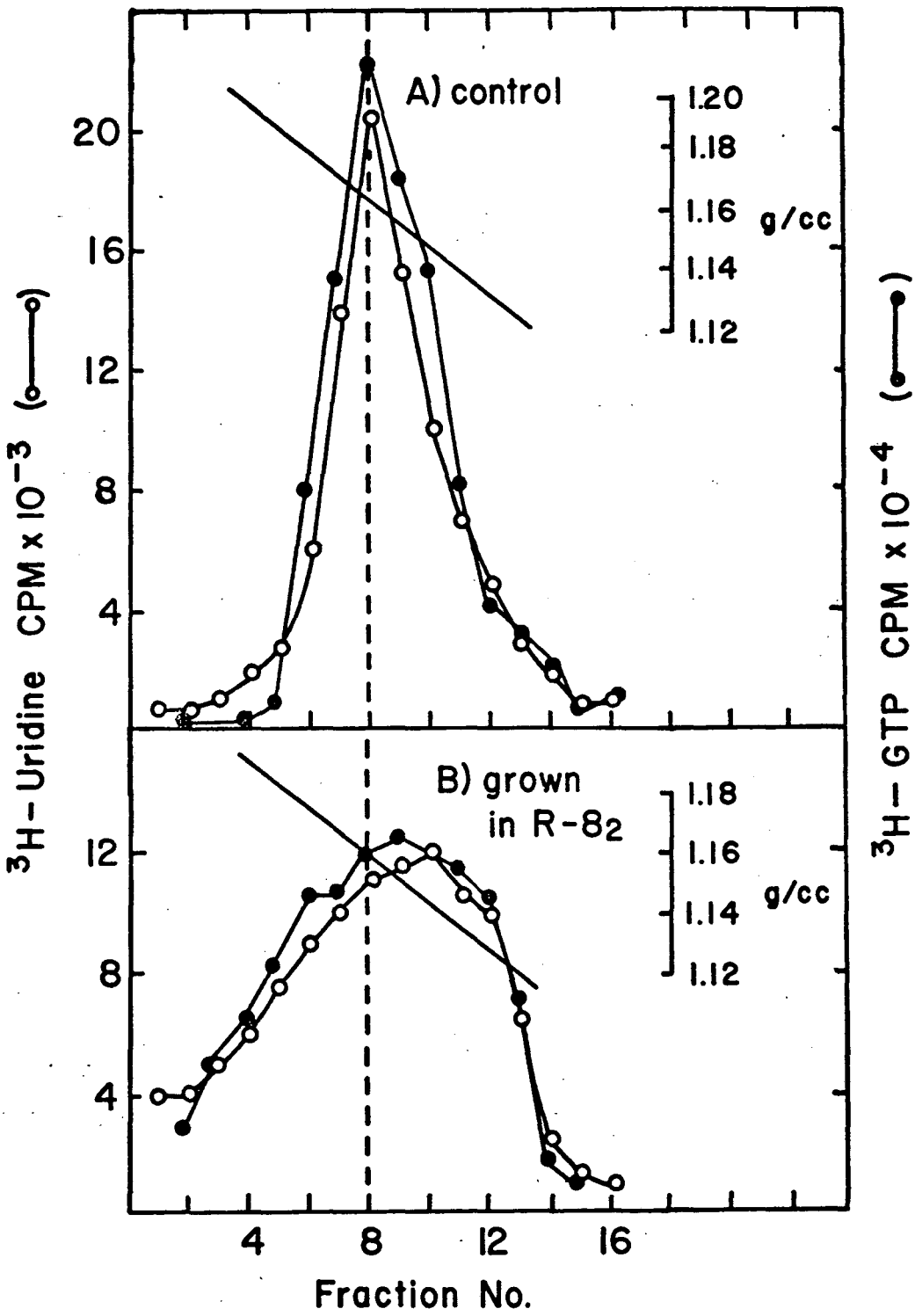

Figure 2

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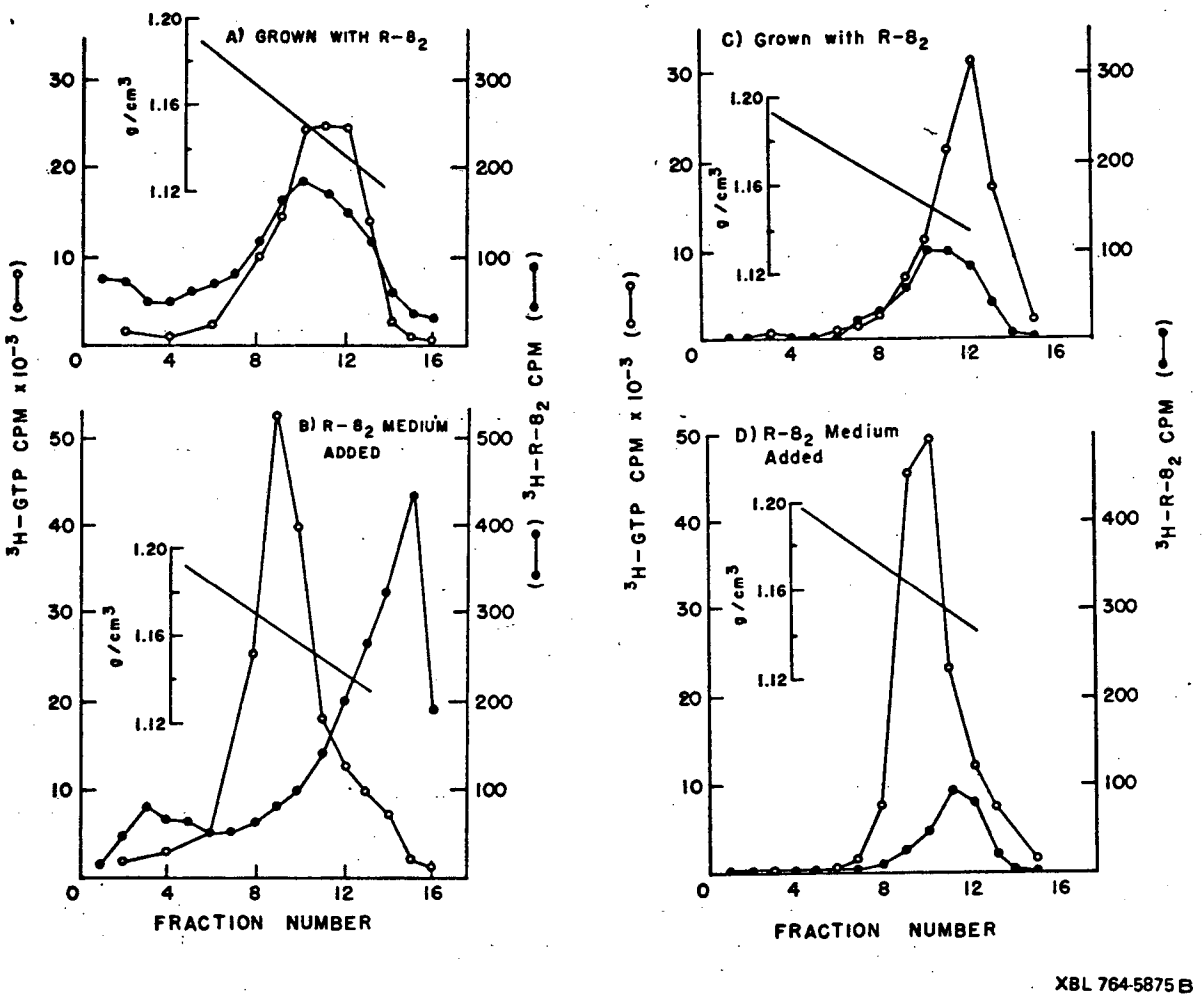

Figure 3

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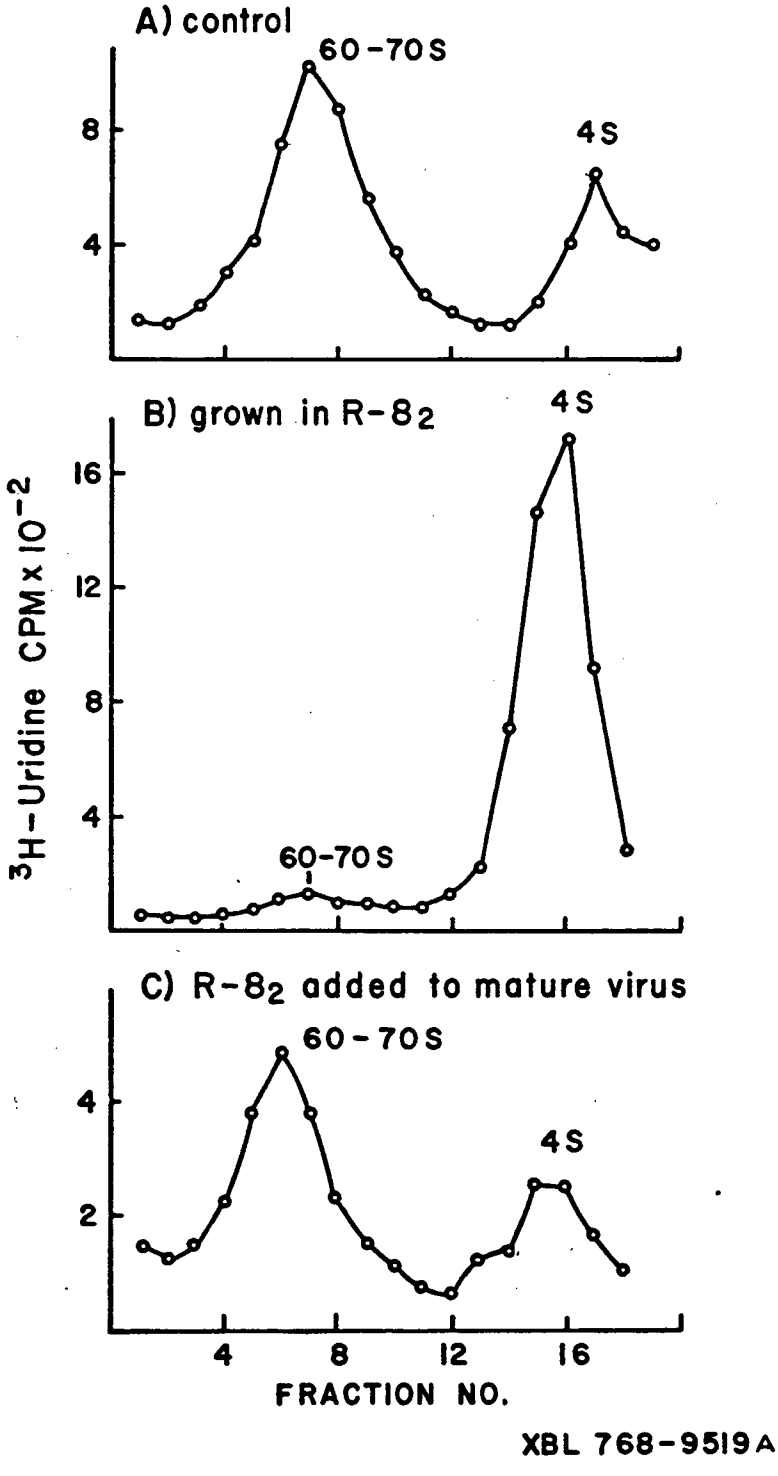

Figure 4

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