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Publication Date
1975-11-01

Submitted to Journal of Virology

LBL-4627
Preprint c.1

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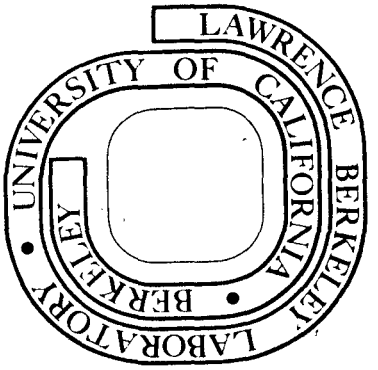
November 1975

Prepared for the U. S. Energy Research and Development Administration under Contract W-7405-ENG-48



DISCLAIMER

INHIBITION OF INFECTIOUS ROUS SARCOMA VIRUS PRODUCTION BY A RIFAMYCIN DERIVATIVE

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ABSTRACT

A new rifamycin derivative, Rifazone-8_2 (R-8_2), an inhibitor of viral RNA-dependent DNA polymerase, is selectively toxic to transformed chick cells in culture. R-8_2 has now been shown to possess antiviral activity as well. The relatively non-toxic properties of R-8_2 have permitted the execution of experiments examining the effect of a rifamycin derivative on virus reproduction. Addition of low concentrations of R-8_2 (15 μg/ml) to cultures soon after Rous sarcoma virus (RSV) infection prevents the spread of infection throughout the culture. This inhibition is not dependent on concomitant cellular transformation since identical results were obtained with cells infected with a transformation-defective RSV. Addition of R-8_2 to cultures in which all the cells are infected does not substantially affect the yield of physical particles as measured by RNA-dependent DNA polymerase activity and by ^{3}H-uridine incorporation into viral RNA. However, the infectivity of the progeny virus, as measured by focus-forming ability, is decreased 95 to 99% by R-8_2 treatment.

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* This work was supported, in part, by the U.S. Energy Research and Development Administration and, in part, by the National Cancer Institute, Grant No. NCI-1-RO-CA-14828-3.

INTRODUCTION

Rifamycin and its derivatives are potent inhibitors of DNA-dependent RNA polymerase of Escherichia coli and several other bacteria (11,29,30). Suitable modifications of the rifamycin molecule produce derivatives which can effectively inhibit the enzymatic action of RNA-dependent DNA polymerase of RNA tumor viruses (6,8,9,31). Ting et al. have shown that preincubation of murine leukemia-sarcoma virus with high concentrations of certain rifamycin derivatives inhibits focus formation (24). The focus inhibitory activity of the derivatives correlated with their ability to inhibit viral RNA-dependent DNA polymerase in vitro. They therefore suggested that rifamycin derivatives inhibit transformation by inhibiting the viral enzyme. However, the presence of these rifamycin derivatives in the growth medium caused considerable reduction in cellular proliferation and thus prevented the direct examination of the effect of these compounds on virus infection and reproduction. Therefore, it had not been determined whether rifamycin derivatives block the initiation of viral development by inhibition of viral RNA-dependent DNA polymerase or if they interfere with continued virus replication.

A number of new rifamycin derivatives synthesized in this laboratory have been shown to be inhibitors of murine leukemia virus RNA-dependent DNA polymerase (23). One of these compounds, rifazone-8_2 (R-8_2) also selectively inhibits the growth of RSV-transformed chick embryo cells in culture and prevents virus-induced focus formation (2).The low concentrations (3 to 15 μg/ml) at which R-8_2 inhibits transformed cell functions permit the continued growth of normal cells (2). It is this

combination of properties which permits the continuous presence of R-8_2 in the cultures and thus allows the study of its effect on virus reproduction.

To aid in determining the effect of R-8_2 on virus production, experiments were performed with cells infected with a transformation-defective mutant of RSV (15,28) as well as with cells infected with the transforming virus of the same sub-group. Cells infected with the transformation-defective strain produce nearly the same amount of virus as transformed cells but are morphologically normal and are as resistant to R-8_2 as normal cells.

We can now report that R-8_2 treatment limits the spread of virus infection when added to cultures in which only a fraction of the cells are initially infected. Furthermore, addition of R-8_2 to cultures in which all the cells are infected does not affect the yield of physical particles but results in a 95 to 99% reduction in the infectivity of the progeny virus.

MATERIALS AND METHODS

Cells and viruses:

Primary cultures were prepared from 10-day old C/O SPF chick embryos as described previously (2,3,19). Prague C Rous sarcoma virus (PR-C RSV) and a transformation-defective derivative, td-PR-C 5431, were used in these studies. Cells were generally infected at a multiplicity of 0.05 focus-forming units (FFU) of PR-C per cell at the time of primary seeding. In parallel cultures, equivalent amounts of td PR-C virus stocks were added. For some experiments, as indicated in the figure legends, cells were infected after the secondary seeding, 4 days after the preparation of primary cultures. Secondary cultures were seeded at 2.5×10^6 cells per 60 mm dish in 5 ml of medium. The medium was removed after 4 hours and virus was added in a total of 0.5 ml. The virus-containing medium was removed after 1 hour and fresh medium added.

Growth of cells in the presence of R-8_2:

All experiments were performed with secondary cultures. For cells infected at the time of primary seeding, R-8_2 (dissolved in DMSO, 15 µg/ml final concentration) was added 4 hours after secondary seeding in medium 199 containing tryptose phosphate broth (2%), calf serum (2%), heated chick serum (1%) and 0.011 M glucose. Cultures infected after secondary seeding were treated with R-8_2 1 hour after the addition of virus. Infected control cultures were prepared in the same manner except that 0.015% DMSO was added. In addition, uninfected R-8_2 treated and untreated chick cells were prepared. Every 24 hours the virus-containing media were collected and stored at -70°C and were replaced with fresh media with R-8_2 or DMSO. Cell growth was monitored by counting sister cultures in duplicate in a Coulter counter.

Preparation and assay of RNA-dependent DNA polymerase:

DNA polymerase was solubilized from PR-C RSV by treatment with 0.5% Triton X-100 (4). The enzyme was purified on a 20 to 40% (V/V) glycerol gradient (5) and stored at -20°C in a solution of 50% glycerol.

The standard assay mixture contained 0.05 M Tris (pH 8.0), 0.05 M KCl, 0.006 M magnesium acetate, 5 mM dithiothreitol, 0.0015% Triton X-100, 0.02 mg/ml template-primer (either poly rC:oligo dG or poly rA:oligo dT) and the appropriate dNTP (either ^{3}H-GTP or ^{3}H-TTP) at a concentration of 0.02 mM and a specific activity of 1 Ci/mmole and 25 µl of diluted enzyme solution. The assays were incubated at 37°C for 1 hour and terminated by the addition of 5% TCA containing 0.01 M sodium pyrophosphate.

For inhibition studies rifamycin derivatives (10 mg/ml) were dissolved in DMSO and were diluted to the desired concentrations before addition to the reaction mixture. The reactions were always initiated by the addition of the enzyme, allowing no pre-incubation with the inhibitors.

Estimation of virus production by measurement of RNA-dependent DNA polymerase activity:

The media collected from growth experiments were centrifuged at 8000 x g for 10 minutes to remove cell debris. Virus was pelleted by centrifugation at 25,000 rpm in the Spinco #30 rotor for 1.5 hours. The pellets were resuspended in standard buffer (4) (0.01 M Tris, pH 7.4, 0.1 M NaCl and 0.001 M EDTA). A 10 µl amount of the resuspended virus preparation was added directly to the DNA polymerase reaction mixture and the enzymatic activity was solubilized by the inclusion of 0.2% Triton X-100 in the assay. Direct measurement of DNA polymerase activity in the growth medium of infected cells yielded results which were comparable to those obtained from the resuspended pellets. Both methods of measurement were routinely used.

Focus assays:

After removal of residual R-8_2, resuspended virus preparations from growth experiments (described above) were diluted from 10^1 to 10^5-fold and the number of focus-forming units (FFU) in each sample was determined as previously described (2,20).

Labeling of viral RNA:

Cultures of virus-infected cells were labeled by the addition of ^{3}H-uridine (New England Nuclear, 39 Ci/mmole) at a final concentration of 20 μCi/ml. After 8 hours the medium was collected and labeled virus particles were analyzed by sucrose density centrifugation.

Virus purification:

Virus particles were pelleted from clarified medium as described above. After resuspension in standard buffer, virus particles were subjected to isopycnic centrifugation (2 hours at 45,000 rpm in the Spinco SW 50.1 rotor) in a 20 to 55% sucrose gradient prepared in standard buffer. The gradients were fractionated and labeled virus was precipitated with TCA, collected on Millipore filters and quantified by liquid scintillation counting.

Rifamycin derivatives:

The synthesis of the rifamycin derivatives has been described previously (25,26). The names of the derivatives were abbreviated as follows: DMB, 2',6'-dimethyl-4'-benzyl-4'-desmethylrifampicin; R-8_2, or Rifazone-8_2, rifaldehyde-N-N-di-n-octyl hydrazine-hydrazone.

RESULTS

Inhibition of Rous sarcoma virus DNA polymerase by rifamycin derivatives

The capacity of R-8_2 to inhibit RSV focus formation has been described (2). While small variation due to sub-group specificity of the virus was observed, direct addition of 10 to 15 µg/ml of R-8_2 to the agar-medium inhibited the number of visible foci by more than 95%. The degree of this inhibition was dependent on the time at which the foci were scored. It was therefore suggested that in addition to possible inhibition of initiation, the growth of the foci was inhibited by additional mechanism. Ting et al. found that pre-incubation of murine sarcoma virus with 100 µg/ml of a number of rifamycin derivatives resulted in greater than 95% inhibition of focus-forming ability of the treated virus (24). In this case, the decrease of viral infectivity was attributed to the concomitant loss of viral RNA-dependent DNA polymerase activity.

The effect of R-8_2 on the enzymatic activity of RNA-dependent DNA polymerase activity partially purified from a transformed line of mouse embryo cells has been reported (23). Since only RSV-infected cells were used in the studies described in this report, the inhibition of RSV DNA polymerase activity by R-8_2 was tested and compared to other rifamycin derivatives (Table 1).RNA-dependent DNA polymerase was purified by glycerol gradient centrifugation from detergent disrupted RSV. The assays were performed in the presence of 0.0015% Triton X-100, a detergent concentration which increases the catalytic activity of the enzyme but does not interfere with the effectiveness of the derivatives

as polymerase inhibitors (22). Of the numerous rifamycin derivatives tested in this laboratory, R-8_2 proved to be one of the most potent inhibitors of RSV DNA polymerase. The inhibition of enzyme activity is relatively independent of the template-primer used in the assay (Table 1), suggesting that the drug acts directly on the polymerase.

Pre-incubation of Rous sarcoma virus with R-8_2

The study of the effect of R-8_2 on virus reproduction required the presence of the derivative in the culture medium. Thus the virus particles synthesized during the course of the experiment would be extruded into the medium and remain in contact with R-8_2. Since R-8_2 was found to be a powerful inhibitor of the viral DNA polymerase, it was essential to determine the effect of R-8_2 on the infectivity of the RSV virions.

Prague C RSV (PR-C RSV) was incubated with either 15 or 100 µg/ml of R-8_2 in growth medium at 37°C. At the specified times, the virus was diluted 10^2- to 10^4-fold and used to infect the cells. After 1 hour of adsorption the virus was removed and the infected cells were overlaid with agar as described (2,20). The effect of pre-incubation of PR-C RSV with R-8_2 on the subsequent focus-forming ability of the virus is shown in Table 2. Pre-incubation with 15 µg/ml of R-8_2 for 5 minutes had no effect on the virus infectivity, and after 1 hour of treatment the virus titer showed a slight increase. Even after 6 hours of pretreatment, the presence of 15 µg/ml R-8_2 had no additional effect on virus infectivity (C. Szabo, unpublished data). However, a significant loss of viral infectivity was seen after pre-incubation with 100 µg/ml of R-8_2 for 5 minutes, and after 1 hour in the presence of the drug, the focus-forming ability

of the virus was inhibited by 99%. The harmless effect of 15 μg/ml of R-8_2 on intact virus simplifies the interpretation of experiments examining RSV reproduction in the presence of the drug.

R-8_2 at a concentration of 15 μg/ml inhibited viral DNA polymerase activity by greater than 95% (Table 1 and unpublished results), blocked focus-formation when added directly to the agar-medium (2), but had negligible effect on viral infectivity with pre-incubation (Table 2). The profound effect on infectivity at high concentrations (100 μg/ml) of R-8_2 may reflect inhibition by other mechanisms, such as disruption of virus particles.

Effect of R-8_2 on the growth of cells infected with a transformation-defective PR-C RSV

Since R-8_2 limits the growth of transformed cells (2), the actual effect of the derivative on virus reproduction may be difficult to determine in transformed cultures. However, cells infected with a transformation-defective strain of virus, td-PR-C, produce progeny virus at nearly the same rate as PR-C transformed cells but are as resistant to R-8_2 as normal cells (Fig. 1). After two days of R-8_2 treatment, normal and td PR-C cells showed only a negligible decrease in growth rate. An additional 24 hrs of drug treatment resulted in only a 20% decrease in cell number compared to control cultures. The PR-C transformed cells, however, showed a decrease in growth rate after two days of drug treatment and a substantial inhibition of growth after three days. Therefore, td PR-C infected cultures can be grown in the presence of R-8_2 and the effect on virus reproduction can be measured with minimal side effects on cellular function. The cytotoxicity of many other rifamycin derivatives requires that experiments be performed by pre-incubation of the virus with the drug before infection of the

cells (24). Pre-incubation experiments can only measure the effect of the derivative on virus infectivity, not on viral reproduction.

Effect of R-8_2 addition one hour after virus infection

The following set of experiments was designed to determine the effect of R-8_2 on virus production. Cell cultures were infected with either PR-C RSV, or the transformation-defective derivative, td PR-C, and grown in the presence of 15 µg/ml of R-8_2. The virus-containing medium was collected every 24 hours and stored at -70°C, and fresh medium containing R-8_2 was added. Virus production during the 24 hour intervals was monitored in three ways: [1] determination of the number of focus-forming units, [2] assay of the viral RNA-dependent DNA polymerase activity and [3] measurement of the viral RNA labeled with ^{3}H-uridine. To correct for different rates of cellular proliferation, all experimental results are presented on a per cell basis.

Secondary cultures of cells were exposed to PR-C RSV for one hour. The virus-containing medium was removed and fresh medium containing 15 µg/ml of R-8_2 was added. Medium was collected and changed every 24 hours. As shown in Table 3, there was a two-fold reduction in focus-forming titer after one day of R-8_2 treatment and by three days infectious virus production was inhibited by greater than 99%.

To test whether non-infectious particles were being produced, or if physical particle production itself had been reduced, the culture medium was assayed for RNA-dependent DNA polymerase activity. To eliminate interference by R-8_2 in the assays, virus particles were collected by centrifugation and resuspended in Tris buffer. Furthermore, the enzyme assays were performed in the presence of 0.2% Triton X-100, a detergent concentration which prevents all R-8_2 inhibition of enzyme activity (22). To avoid the effects of a reduced rate of cellular proliferation (24) on

progeny particle production, identical growth experiments were also performed with cells infected with the transformation defective virus described above. As illustrated in Fig. 2, after three days virus particle synthesis in the presence of R-8_2 was only 15% that of the control cultures. The extent of inhibition of physical particle production was nearly identical for cells infected with either PR-C (Fig. 2a) or td PR-C RSV (Fig. 2b).

R-8_2 can effectively prevent the spread of viral infection when added to cultures at the time of virus addition and this inhibition is not dependent on viral transformation. However, this experiment did not discriminate between the prevention of secondary infections or the inhibition of the initiation of viral development within the cell. Nevertheless, the experimental results indicated that R-8_2 had an additional effect on the synthesis of infectious virus. After three days of R-8_2 treatment, the production of infectious virus was decreased 100-fold (Table 2) while physical particle production was decreased only 10-fold (Fig. 2).

Effect of R-8_2 on virus reproduction in infected cultures

Infection of cells with either PR-C RSV or td PR-C at low multiplicity at the time of primary seeding yields cultures in which approximately 50% of the cells are infected at the time of secondary seeding four days later. By two days after secondary seeding nearly all the cells in culture are infected or morphologically transformed. Since at this stage the reinfection process plays almost no role in the rate of virus production, the determination of virus synthesis, after the addition of R-8_2 to such cultures, measures the effect of the derivative on viral replication.

Addition of 15 μg/ml of R-8_2 two days after secondary seeding of PR-C transformed cells resulted in a reduction in the focus-forming titer of the progeny virus to only 6% of that of control cultures after 24 hours of treatment (Fig. 3a). A twenty-fold decrease in the focus-forming titer was maintained after the second and third days of drug treatment.

On the other hand, R-8_2 treatment reduced PR-C virus particle production by only 10% after 24 hours and 50% after 48 hours (Fig. 3a). The decreased rate in PR-C production after two and three days of R-8_2 treatment probably reflects the toxic effect of the drug on the transformed cells, since treatment of cultures identically infected with td PR-C resulted in almost no change in the synthesis of progeny virus particles (Fig. 3b). As in other experiments, the results presented in Fig. 3 were corrected for cell number. However, it is not clear what role the reduced metabolic rate of dying cells may play in the level of virus production.

It appears that R-8_2 prevents the synthesis of infectious virus particles and thereby inhibits the initiation of new infections. If this were the case, then the addition of R-8_2 to mixed cultures of infected and uninfected cells should prevent the infection of the remaining normal cells in the culture without affecting virus particle production in cells infected before R-8_2 addition. To test this hypothesis, the ability of R-8_2 to limit virus production in mixed cultures was examined. Cells were infected at low multiplicity at the time of primary seeding and R-8_2 was added after the secondary seeding four days later. Under these conditions, approximately 40 to 50% of the cells in culture appeared morphologically transformed at the time of R-8_2 addition. Untreated cultures showed a continuous increase in

the production of virus particles throughout the course of the experiment (Fig. 3). As expected, virus particle production in the R-8_2 treated cultures appeared to remain relatively constant during the three days of drug treatment.

As in previous experiments the focus forming titer of progeny virus was drastically reduced by R-8_2 treatment (Fig. 4a). A reduction of more than 95% in infectious particle synthesis was detected by 24 hours after R-8_2 addition while physical particle production was only slightly affected at that time (Fig. 4a).

The measurement of viral RNA-dependent DNA polymerase activity generally yields a good estimation of the relative amount of virus particles. However, since R-8_2 is an inhibitor of this enzyme, an additional technique was employed for studying the effect of the drug on virus particle production. Infected cultures were labeled with ^{3}H-uridine in the presence or absence of R-8_2 and the synthesis of labeled virus was monitored by sucrose density centrifugation.

Mixed cultures were infected and treated with R-8_2 as described above. After 48 hours of treatment with R-8_2, cultures were labeled for eight hours with ^{3}H-uridine. The inhibition of virus particle production by R-8_2 as measured by ^{3}H-uridine incorporation (Fig. 5) was comparable to the decrease in viral DNA polymerase activity (Fig. 4). The total amount of ^{3}H-label recovered at the buoyant density (1.16 - 1.18 g/cm^3) of the virus was reduced approximately 3-fold after R-8_2 treatment of PR-C transformed and two-fold after treatment of td PR-C infected cells (Fig. 5). The physical particle inhibition observed by measuring the viral RNA-dependent DNA polymerase activity was 3.4- and 1.7-fold, respectively (Fig. 4).

DISCUSSION

Rifazone-8_2, a rifamycin derivative which preferentially inhibits the growth of virus transformed chick cells in culture, has now been shown to possess antiviral activity as well. The relatively low toxicity of R-8_2 to non-transformed cells has made it possible to examine the effect of a rifamycin derivative on virus replication. Although R-8_2 is toxic to transformed cells, cultures infected with a transformation-defective RSV proliferate nearly as well as normal cells in the presence of the derivative. R-8_2 prevents the spread of infection when added to cultures in which only a fraction of the cells are infected. Studies with the transformation-defective virus indicate that the inhibitory action of the drug on the virus does not require concomitant cell transformation. There appears to be virtually no inhibition of physical particle production by the derivative in cultures with established virus infections, except in transformed cultures in which the cells are dying from the effect of the drug.

R-8_2 is a potent inhibitor of viral RNA-dependent DNA polymerase, an enzyme essential for the establishment of RNA tumor virus infection (16). Ting, et al. (24) have observed for a number of rifamycin derivatives a direct correlation between inhibition of viral DNA polymerase activity and inhibition of focus formation of murine leukemia-sarcoma virus. These studies were performed by pre-incubation of the virus with high concentrations (100 µg/ml) of the derivatives and removal of the derivatives by washing and dilution before testing the focus forming titer and DNA polymerase activity of the treated virus. These workers suggested that the inhibition of the viral DNA polymerase was responsible for the observed focus inhibition. However, the interpretation of such experiments

is difficult in the light of the work of Gurgo, et al. (27) who have shown that rifamycin inhibition can be completely reversed by dilution of the drug-enzyme mixture. Furthermore, experimental results presented in this report indicate that pre-incubation with R-8_2, an extremely powerful inhibitor of RSV DNA polymerase, had no adverse effect on viral infectivity at a concentration of 15 µg/ml. On the other hand, pre-incubation with 100 µg/ml of R-8_2 caused a severe reduction in the subsequent focus-forming ability of the virus. This latter may be due to a non-specific inactivation or disruption of the virus particles by very high concentrations of the rifamycin derivatives.

When added soon after infection, R-8_2 does indeed prevent focus formation and the spread of viral infection, possibly by the inhibition of the viral DNA polymerase. However, the major anti-viral effect of R-8_2 seems to result from the loss of infectivity of the progeny virus particles. The resulting synthesis of non-infectious PR-C RSV appears to be an action of R-8_2 on processes which are distinct from inhibition of RNA-dependent DNA polymerase activity. Addition of R-8_2 to cultures in which all the cells are transformed causes almost no decrease in particle production during the first 24 hours of drug treatment but results in a 95 to 99% reduction in focus-forming titer when the virus so produced is assayed in the absence of the drug. Since a functional viral RNA-dependent DNA polymerase is not required for continued virus production (16,27), the loss of viral infectivity may then be the result of an alteration of some other viral component.

One explanation for non-infectious virus production is that R-8_2 binds to the polymerase as it is packaged into the virion at concentrations which do not inhibit the enzyme in vitro, but prevent reinfection, and

thus focus formation. For this interpretation to be correct, the derivative must be packaged into the virion during its synthesis since 15 µg/ml or R-8_2 has no effect on intact virions. Also, the binding of R-8_2 to (or in) the virus particle must be extremely tight because the virions are washed and diluted before the assay of focus-forming ability.

Other possible mechanisms for non-infectious virus production could include interference with the assembly of the infectious virus. Glucosamine and 2-deoxyglucose (2-DG) have been shown to block the maturation of influence virus (12,13) and lead to the accumulation of a non-glucosylated viral polypeptide (14). Recently, Prochownik, et al. (18) have shown that transformed rat kidney cells treated with 2-DG produce murine sarcoma-leukemia virus with structural alterations in virus proteins. The addition of 2-DG does not affect physical particle production but reduces the infectivity of the released virus. It is possible that R-8_2 acts in the manner of an anti-metabolite and causes aberrant synthesis of an essential virus component(s) which leads to the production of non-infectious particles.

High concentrations of rifampicin have been found to inhibit the growth of pox viruses in tissue culture (21). The antiviral effect appears to result from inhibition of late viral protein synthesis (10) as well as interference with virion assembly (17). Alternatively, the non-infectious particle synthesis mediated by R-8_2 may be the result of interference with similar processes in the RSV growth cycle.

In summary, the anti-viral action of R-8_2 may be due, in part, to inhibition of viral RNA-dependent DNA polymerase activity and thus prevention of the initiation of RNA tumor virus infection. However, the most potent action of the derivative is the effect on virus infectivity. This effect on the infectivity of the virus appears to be a property

of R-8_2 which is distinct from the derivative's killing action on transformed cells. It is possible to isolate RSV-variants which can form foci in the presence of R-8_2. However, cells transformed with such virus strains are as sensitive to the preferential killing of R-8_2 as cells transformed with normal RSV. (Szabo, C., unpublished observation). Thus, the conversion of the virus from a sensitive to a R-8_2-resistant form does not alter the drug sensitivity of the infected cell. Experiments are in progress to determine the nature of the virus alteration produced in the presence of R-8_2.

We thank Dr. Edward L. Bennett for his interest in this work. We also thank Dr. Peter Duesberg for providing us with stocks of Prague C RSV and its transformation-defective mutant td PR-C 5431 and Dr. Steven Martin for his critical reading of the manuscript. The indispensable work of Carol Hatie in culturing the cells is gratefully acknowledged.

TABLE 1. Inhibition of RSV RNA-Dependent DNA Polymerase[a]

Derivative	µg/ml for 50% inhibition[b]	
	Template	
	$(rC)_n:(dG)_{12-18}$	$(rA)_n:(dT)_{12-18}$
Rifampicin	>100	>100
DMB	25	40
$R\text{-}4_2$	4.3	8.2
$R\text{-}8_2$	1.9	3.5
$R\text{-}9_2$	3.6	10

a Viral DNA polymerase was purified from detergent-disrupted virions as described in the text.

b Values were taken from concentration-dependent inhibition curves.

TABLE 2. Focus-Forming Ability of PR-C RSV after Pre-incubation with R-8_2

Treatment	Focus number (% of control) 5 min[a]	60 min[b]
Control	100 (64 ± 5)[c]	100 (63 ± 5)[c]
R-8_2 (15 µg/ml)	97	134
R-8_2 (100 µg/ml)	64	1.2

a A stock of PR-C RSV was incubated at 37°C for 5 min in the presence of R-8_2 before dilution and assay of focus-forming titer.

b Same as footnote a except that pre-incubation was for a 60 min period.

c The number in parenthesis indicates the average number of foci from triplicate assays which was set equal to 100%.

TABLE 3. Effect of R-8_2 Addition, One Hour after Infection, on the Synthesis of Infectious PR-C RSV[a]

Days after R-8_2 Addition	FFU Produced/10^6 Cells[c]		% Inhibition
	Control	R-8_2 (15 µg/ml)[d]	
1	1.1×10^3	6.2×10^2	44
2	3.2×10^4	5.3×10^3	83
3	5.1×10^5	4.2×10^3	99.2

[a] Cultures were infected with 0.5 FFU of PR-C RSV per cell 4 hrs after secondary seeding. R-8_2 (15 µg/ml) was added 1 hr after virus infection.

[b] Media were collected and replaced with 15 µg/ml R-8_2 every 24 hrs.

[c] Culture media were titered in duplicate assays and foci were scored after 8 days.

[d] The concentrations of any residual R-8_2 remaining with the virus in the focus assay were too low to affect focus formation.

FIGURE LEGENDS

Fig. 1. Growth of PR-C transformed (A), td PR-C infected (B) and normal (C) cells in the presence of R-8_2. Cells were seeded at 1 x 10^6 cells per 60 mm dish 4 days after primary seeding in medium containing either DMSO (O) or 15 µg/ml R-8_2 (●). On successive days the medium was changed, cells from triplicate plates were removed by trypsinization and cell numbers were determined in a Coulter counter.

Fig. 2. Effect of R-8_2 on viral RNA-dependent DNA polymerase activity after infection of secondary cultures. Four hours after secondary cultures were seeded at 2.5 x 10^6 cells per 60 mm dish, cells were exposed to either PR-C RSV (A) or td PR-C RSV (B) for 1 hr. The virus was then removed and medium containing 15 µg/ml of R-8_2 (●) was added to half the cultures while control cultures received medium with DMSO (O). Every 24 hrs for 3 days the culture media were collected and immediately placed at -70°C and fresh drug-containing media were added. Viral RNA-dependent DNA polymerase activity was determined as described in Materials and Methods. To correct for different rates of cellular proliferation during the course of the experiment, the experimental results are presented as the relative amount of viral DNA polymerase activity produced per a constant number of cells.

Fig. 3. Effect of R-8_2 on virus reproduction in cultures infected with either PR-C (A) or td PR-C (B) RSV at the time of primary seeding. Secondary cultures were prepared 4 days after primary seeding at a density of 1.0 x 10^6 cells per 60 mm dish. Every 24 hrs the supernatant fluids from the infected cultures were collected and fresh media were added. After

collecting the culture media on day 2, half the cultures received media containing 15 µg/ml of R-8_2 (●) and control cultures were grown in media containing DMSO (O). Media were collected and RNA-dependent DNA polymerase activity (——) in each sample was determined after removal of the drug as described in Materials and Methods and in the legend to Fig. 2. The number of focus-forming units (FFU) in each sample (----) was also determined and presented on a per cell basis.

Fig. 4. Effect of R-8_2 on virus reproduction in mixed cultures of normal and PR-C transformed cells (A) and cultures of normal and td PR-C infected cells (B). Cells were infected at the time of primary seeding and treated with 15 µg/ml R-8_2 (●) or DMSO (O) 4 hrs after secondary seeding. Culture supernatants were collected every 24 hrs and assayed for viral RNA-dependent DNA polymerase activity (——) and for FFU (-----) as described in Materials and Methods.

Fig. 5. Effect of R-8_2 on the incorporation of radioactive label into viral RNA. Mixed cultures of normal and PR-C transformed cells (A,B) and cultures of normal and td PR-C infected cells (C,D) were treated with 15 µg/ml R-8_2 (●) or DMSO (O) 4 hrs after secondary seeding. After 2 days of drug treatment, ^{3}H-uridine (20 µCi/ml) was added to the cultures for 8 hrs. Culture fluids were collected, concentrated and analyzed for radioactively labeled virus by sucrose gradient centrifugation as described in Materials and Methods.

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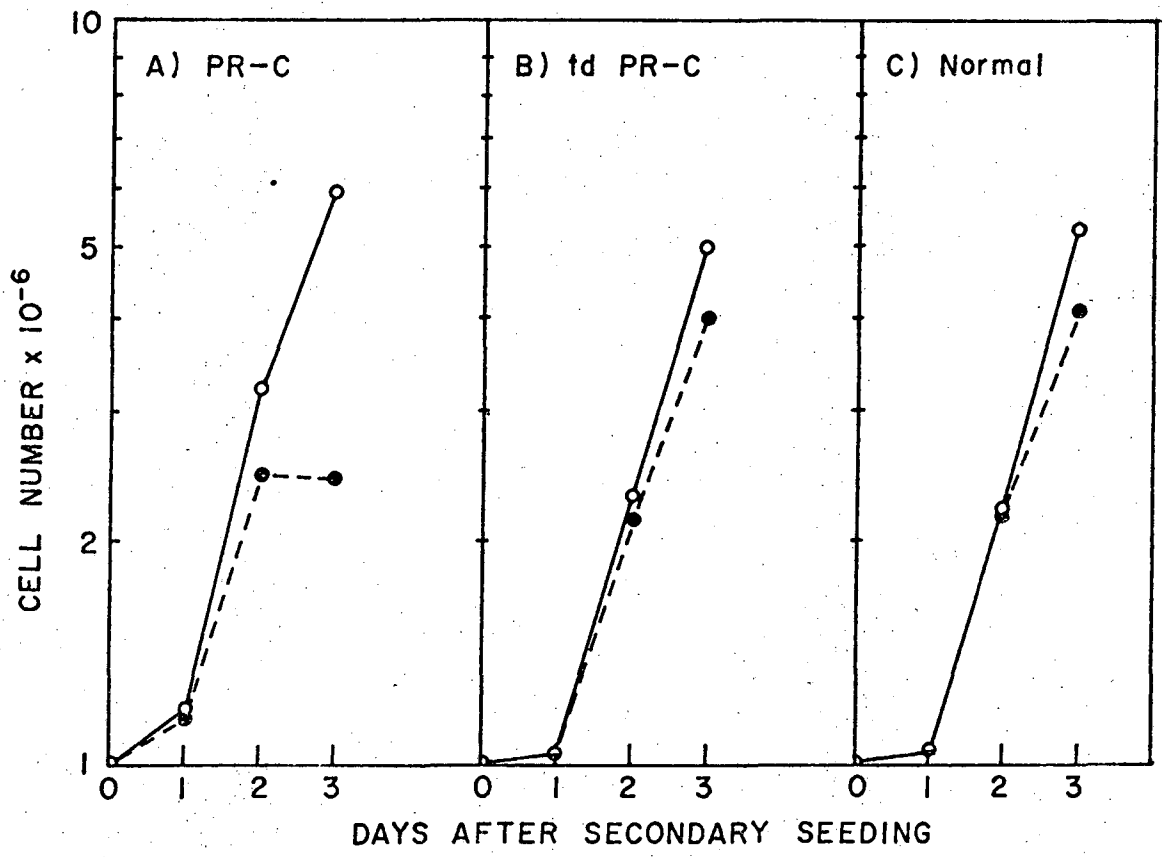

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Fig. 1

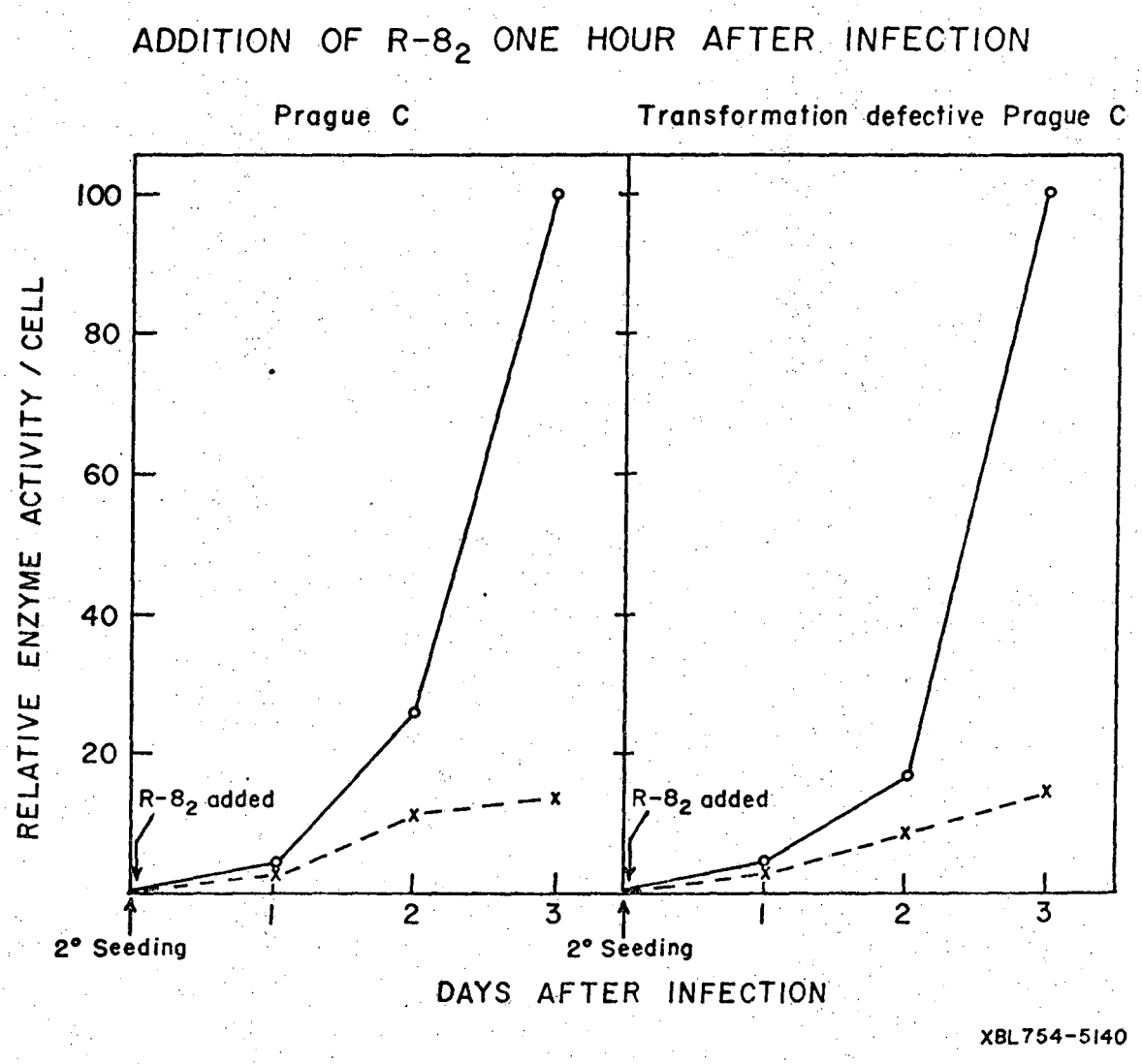
ADDITION OF R-8$_2$ ONE HOUR AFTER INFECTION
Prague C
Transformation defective Prague C
RELATIVE ENZYME ACTIVITY / CELL
100
80
60
40
20
R-8$_2$ added
R-8$_2$ added
2° Seeding
2° Seeding
1
2
3
1
2
3
DAYS AFTER INFECTION
XBL754-5140

Fig. 2

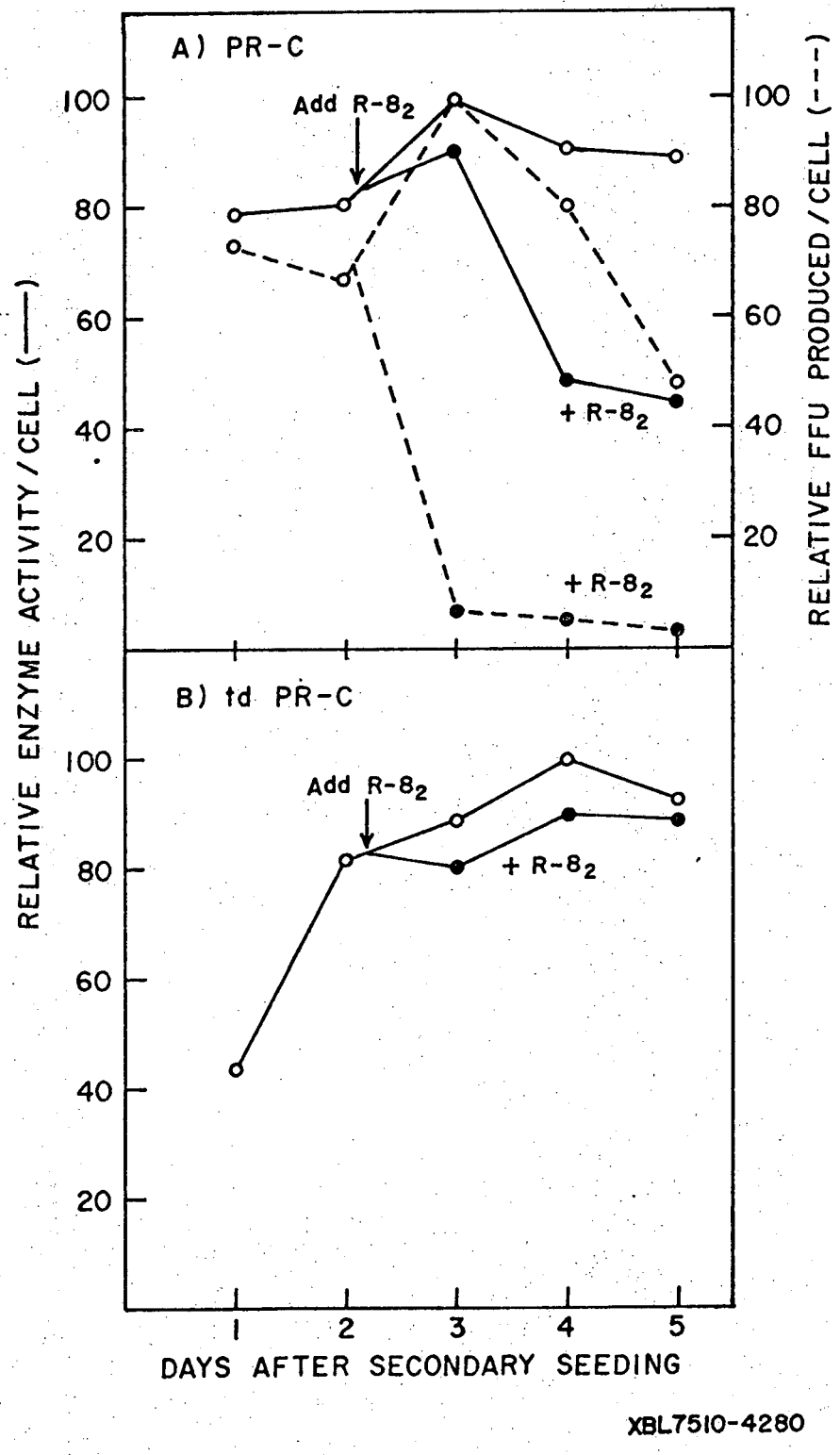
A) PR-C
Add R-8$_2$
+ R-8$_2$
+ R-8$_2$
B) td PR-C
Add R-8$_2$
+ R-8$_2$
RELATIVE ENZYME ACTIVITY / CELL (——)
RELATIVE FFU PRODUCED / CELL (---)
100
80
60
40
20
1
2
3
4
5
DAYS AFTER SECONDARY SEEDING
XBL7510-4280

Fig. 3

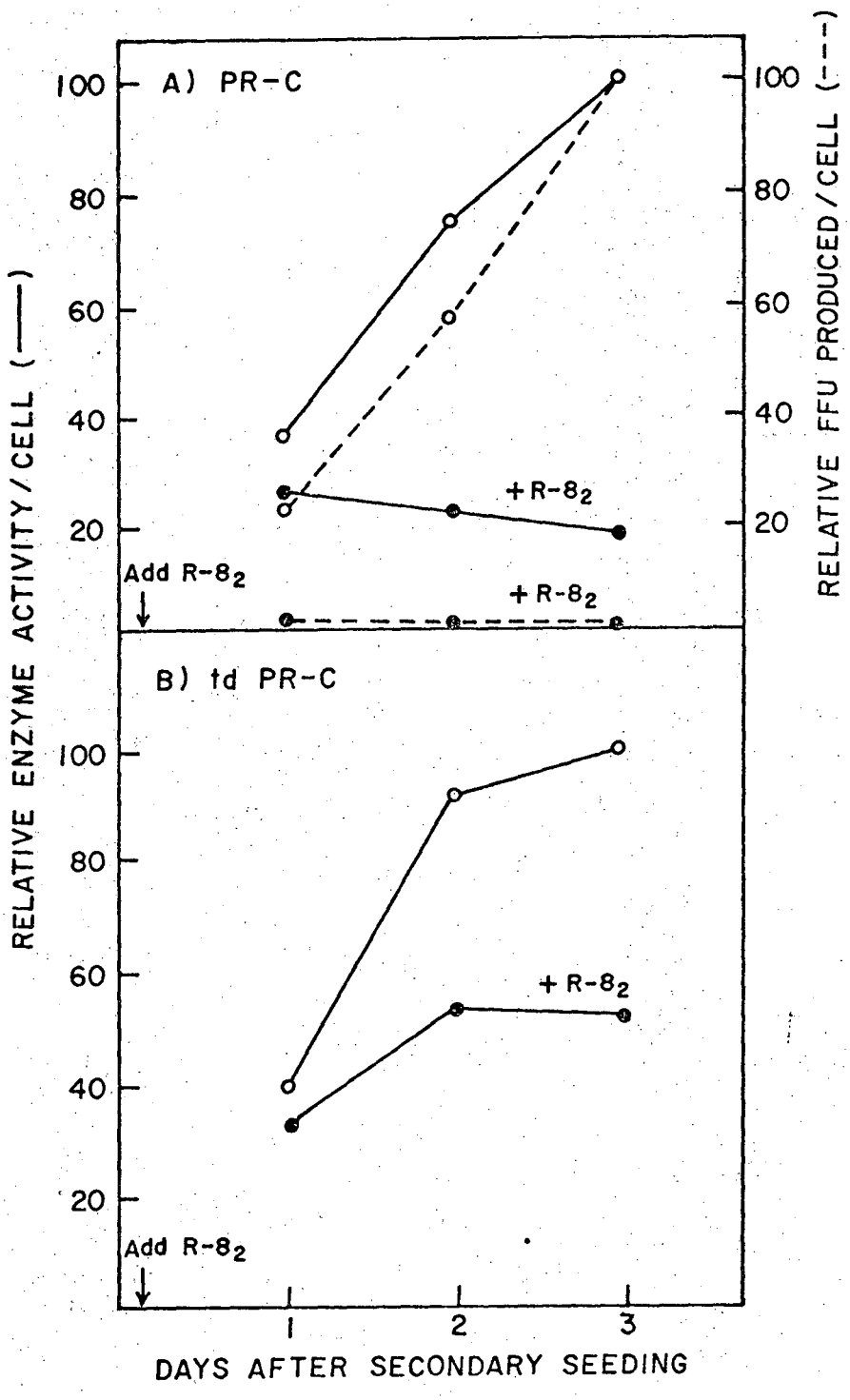
A) PR-C
B) td PR-C
+R-8₂
+R-8₂
+R-8₂
+R-8₂
Add R-8₂
Add R-8₂
RELATIVE ENZYME ACTIVITY / CELL (——)
RELATIVE FFU PRODUCED / CELL (---)
100
80
60
40
20
1
2
3
DAYS AFTER SECONDARY SEEDING
XBL7510-4281

Fig. 4

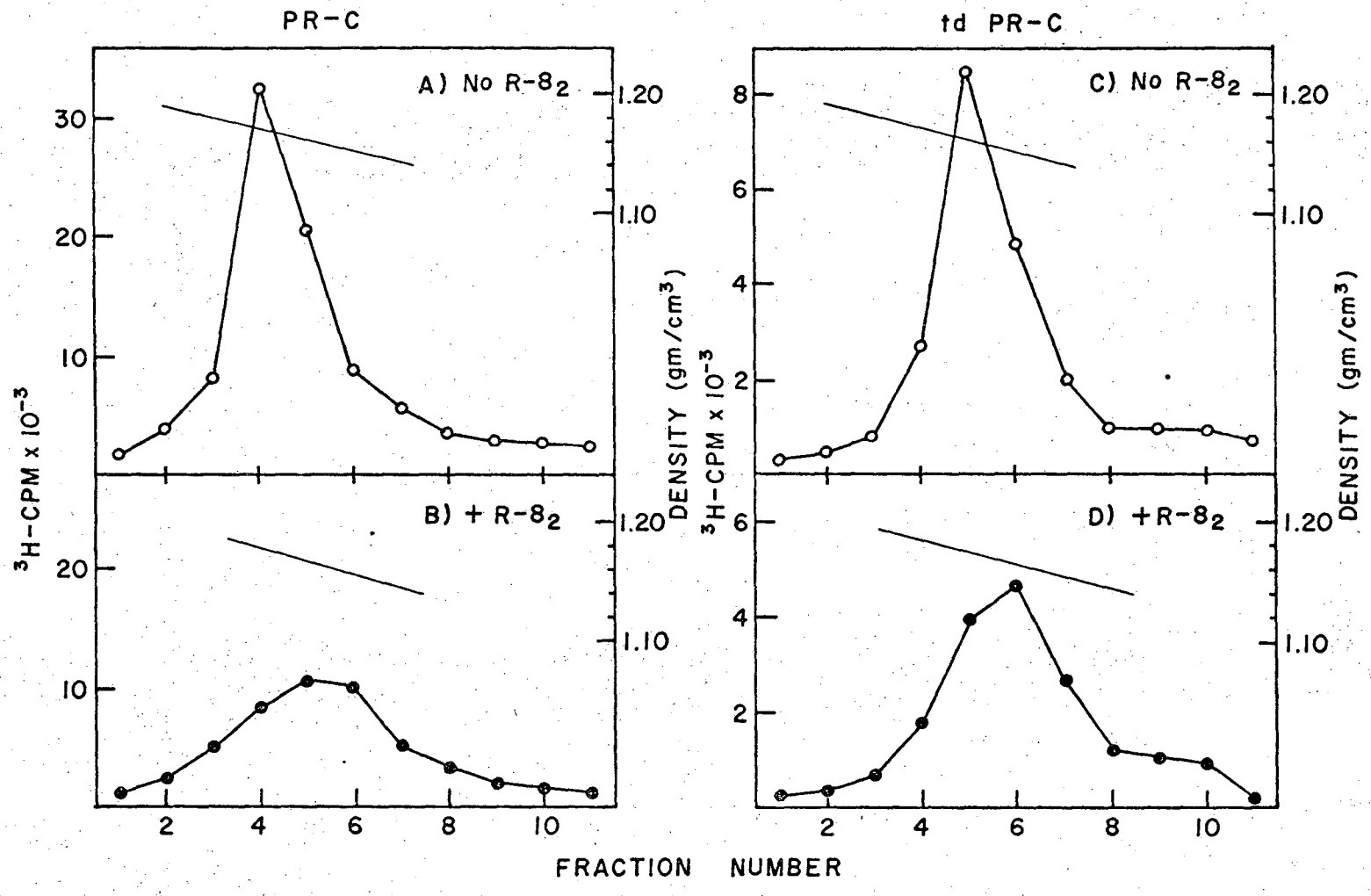

Fig. 5

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