

Preferential Inhibition of the Growth of Virus-Transformed Cells in Culture by Rifazone-8₂, a New Rifamycin Derivative

(selective growth inhibition/virus transformation/chick fibroblasts)

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Contributed by Melvin Calvin, April 1, 1974

ABSTRACT Rifazone-8₂, a new rifamycin derivative, is shown to preferentially inhibit the growth of virus-transformed chick cells in culture. Macromolecular synthesis and glucose uptake of transformed cells are also appreciably decreased in the presence of low concentrations of rifazone-8₂ where the normal cells appear unaffected. While rifazone-8₂ is shown to be a selective inhibitor of RNA-directed DNA polymerase *in vitro*, its action on the growth of transformed cells may involve some other mechanism.

Rifampicin and its derivatives have been shown to inhibit focus formation by RNA tumor viruses in various cell types in culture (1-5). The *in vitro* demonstration that RNA-directed DNA polymerase is inhibited by the action of some of these derivatives (6-13) has raised the possibility that the drugs may inhibit focus formation by inhibiting the activity of RNA-directed DNA polymerase. Smith *et al.* have shown recently that those rifamycin derivatives that are toxic to leukemic human leukocytes are also those that inhibit RNA-directed DNA polymerase best *in vitro* (14).

Rifampicin, a well-known derivative of rifamycin, has been shown to inhibit the replication of vaccinia virus in tissue culture when added at very high levels (>100 µg/ml) (15, 16) and to reduce the incidence of adenovirus-induced tumors in male hamsters (16). In chick cells it has been argued that rifampicin (a) inhibits focus formation by Rous sarcoma virus (RSV) (1), (b) has no effect on transformation, as it is toxic to both normal and transformed cells (17), and (c) is preferentially toxic to transformed cells (20). Variation in culture conditions, serum concentration, and cell density undoubtedly play a role in such contradictory findings. For example, whether or not amphotericin B is present in culture medium may drastically change the result of focus inhibition (4, 19). Furthermore, the high concentration of rifampicin used in these experiments (20-80 µg/ml) under some conditions is quite toxic to normal cells and makes interpretation of these data very difficult. Rifampicin itself has little or no effect on activity of RNA-directed DNA polymerase *in vitro* at concentrations used in tissue culture studies (7, 17, 20). We have tested several new rifamycin derivatives synthesized in this laboratory that have been shown to be inhibitors of RNA-directed DNA polymerase *in vitro* and

inhibitors of focus formation *in vivo* in other cell systems (4-6). In addition, we have tested rifazone-8₂ (R-8₂), a new rifamycin which is to date the most selective inhibitor of viral RNA-directed DNA polymerase *in vitro* (6, 21). We find that at low concentrations (3-10 µg/ml), R-8₂ can selectively inhibit the growth of transformed cells and prevent focus formation while allowing the normal cell growth and function to continue.

MATERIALS AND METHODS

Growth of Cell Cultures. Primary cultures were prepared from 10-day-old C/O or C/B type SPF chick embryos as described (23, 24), except that amphotericin B (Fungizone) was eliminated at this point (25). The cells were seeded in medium 199, which was supplemented with tryptose phosphate broth (2%), calf serum (1%), and heated chicken serum (1%). The medium was changed on day 3. Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 60- or 35-mm petri dishes. An additional 1 mg/ml of glucose was added to the medium at this time, bringing the final concentration of glucose to 11.00 mM, and calf-serum concentration was raised to 2%. For studies with transformed cultures, half the cells of a single embryo were infected 4 hr after primary seeding with 4×10^6 focus-forming units of SR-RSV or B-RSV. Secondary cultures were prepared as above.

Focus Assay. Assays were performed essentially as described (22), with slight modifications. We found that gentle removal of the agar on day 4 or 5 and addition of either liquid medium or another agar overlay enhances the visibility of foci. Four hours after secondary normal cells were seeded at 2.5×10^6 per 60-mm dish, medium was removed and cells were exposed to the appropriate dilution of virus in 0.5 ml of medium for 1 hr. The virus was then removed, the monolayer was rinsed, and appropriate concentrations of derivatives were added together with 1 µg/ml of Fungizone in 0.5 ml of medium 199. Rifamycins were dissolved in dimethyl sulfoxide [(Me)₂SO] so that all cultures had a final concentration of 0.1% (Me)₂SO. Thirty minutes later the derivatives were removed and cells were overlaid with agar-medium containing the same concentration of the derivatives. In experiments where the rifamycins were added at a later time, the agar layers of control cultures were removed and replaced with agar containing (Me)₂SO at the same time. A known titer of RSV stock accompanied all assays.

Abbreviations: R-8₂ or rifazone-8₂, rifaldehyde-*N,N*-di-*n*-octyl hydrazine-hydrazone; RSV, Rous sarcoma virus; B-RSV, Bryan-high-titer strain of RSV; SR-RSV, Schmidt-Ruppin subgroup A strain of RSV; Rif, Rifampicin; DMB, 2',6'-dimethyl-4'-benzyl-4'-desmethylrifampicin; (Me)₂SO, dimethyl sulfoxide.

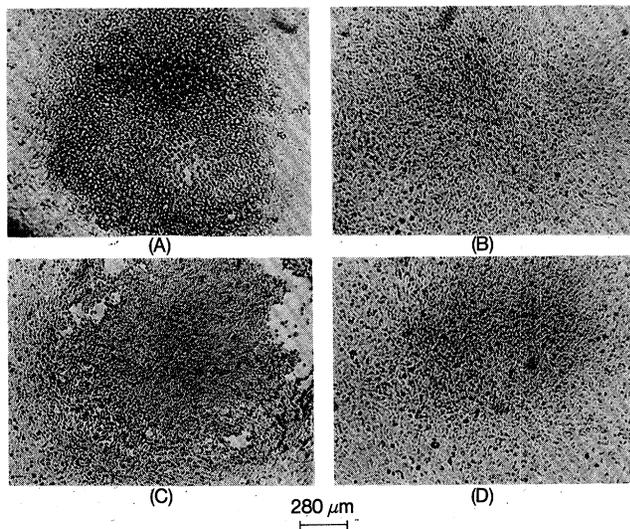


FIG. 1. Focus formation in the presence and absence of R-8₂. Focus of SR-RSV on day 8. (A) in 0.1% (Me)₂SO; (B) in the presence of R-8₂ (10 µg/ml) added 1 hr after virus infection; (C) in 0.1% (Me)₂SO; the agar overlay was replaced after 2 days; (D) in the presence of R-8₂ (10 µg/ml) added 2 days after virus infection.

Measurements of the Rate of DNA and Protein Synthesis and Glucose Uptake. Radioactive assays were performed as described (25, 26). All radioactive compounds were purchased from New England Nuclear Corp.

Rifamycin Derivatives were synthesized as described (20, 21).

RESULTS

Focus Formation. When foci were scored on day 8 and 6 for SR-RSV or B-RSV, respectively, there was a marked inhibition of the number of foci in the presence of rifazone-S₂ (Table 1). Dimethylbenzyl rifampicin (DMB) (10 µg/ml) also caused appreciable inhibition with SR-RSV. Rifazacyclo-16 (RC-16), previously shown to be an inhibitor of murine sarcoma virus focus formation on UCL-B cells (4), was not very effective in chick cells. Rifampicin, at 20 µg/ml,

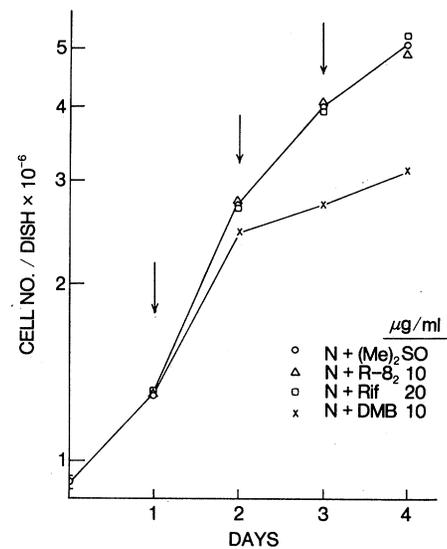


FIG. 2. Growth of high-density normal cells in the presence of rifamycin derivatives. Normal cells (*N*) were plated at $1 \times 10^5/\text{cm}^2$ in the presence or absence of the derivatives as described in *Methods*. Average of four measurements. Values on *ordinate* have been multiplied by 10^{-6} , as indicated. *Arrows* indicate days on which the medium was changed.

had no effect on focus formation. The few remaining foci in the presence of the effective rifamycins were usually much smaller than the control foci (Fig. 1). When the foci were scored again 3 days later, the apparent inhibition had decreased in almost all cases, suggesting an inhibition of the growth of the focus once it had been formed (Table 1).

Normal Cells at High Cell Density. Under the condition of the agar assay (more than 1×10^5 normal cells per cm^2), 10 µg/ml of R-8₂ had no effect on the growth properties of normal cells (Fig. 2). DMB, however, was toxic under these conditions. To avoid the complication of toxicity under assay conditions, we chose to work with R-8₂ alone. The pronounced inhibition of focus formation by R-8₂, therefore, is not due to toxicity to the normal cells in the monolayer. As a further control, the normal cells from the uninfected areas of R-8₂ cultures were

TABLE 1. RSV focus inhibition by rifamycins (% of control)

	Schmidt-Ruppin*		Bryan†	
	Day 8	Day 11	Day 6	Day 9
Control [(Me) ₂ SO]	100 (80 ± 20)‡	100 (85 ± 20)	100 (70 ± 20)	100 (70 ± 20)
Rifampicin (20 µg/ml)	100 ± 10	100 ± 10	100 ± 10	100 ± 8
RC-16 (10 µg/ml)	75 ± 10	85 ± 10	—	—
DMB (1 µg/ml)	50 ± 7	85 ± 8	—	—
DMB (10 µg/ml)	0-2	10 ± 6	—	—
R-8 ₂ (1 µg/ml)	90 ± 10	90 ± 10	90 ± 10	95 ± 10
R-8 ₂ (5 µg/ml)	40 ± 10	70 ± 15	45 ± 10	70 ± 15
R-8 ₂ (10 µg/ml)	0-1	15 ± 10	10 ± 8	20 ± 10

Focus-forming units (50-100) of Schmidt-Ruppin subgroup A or Bryan strain of Rous sarcoma virus were assayed under agar as described in *Methods*. The foci were scored on days 8 and 11 for SR and on days 6 and 9 for the Bryan strains. Amphotericin B (1 µg/ml) was present throughout the focus assay, even though at best it improved the inhibition by only 10%.

* Average of six experiments.

† Average of four experiments.

‡ The number in parentheses indicates the actual number of foci, which was set equal to 100%.

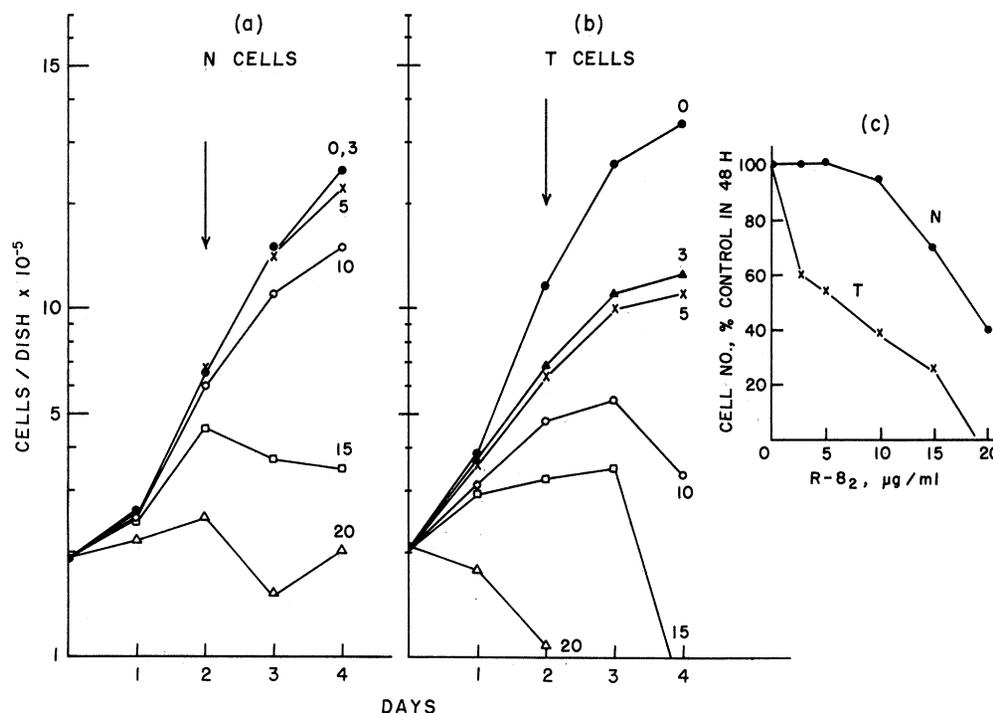


FIG. 3 Growth of normal and RSV-transformed cells in the presence of R-8₂. Normal (a) and SR-transformed (b) cells were seeded at 2×10^4 cells per cm² 4 days after primary seedings. The medium contained either (Me)₂SO or R-8₂ (3, 5, 10, and 20 µg/ml). Cells were counted on successive days in triplicate. (c) The percent cells left on the dish after 48 hr (taken from a and b; the cell number in control cultures was set equal to 100). N, normal; T, transformed.

removed from under agar and replated. They showed normal growth properties when compared to control cultures regrown after agar removal and were morphologically normal as well.

Comparison of Normal and Transformed Cells at Low Densities. When cells were seeded at lower cell densities (2×10^4 cells per cm²), they were more sensitive to R-8₂ than cells at the higher cell densities (compare Figs. 2 and 3a). However, at comparable cell densities and growth rates, transformed cells were always more sensitive than normal cells (Fig. 3). While normal cells were unaffected by 5 µg/ml of R-8₂, the growth of transformed cells was considerably inhibited by 3 µg/ml of R-8₂ after 48 hr (Fig. 3c). Three days after addition of 5 µg/ml of R-8₂, the morphology and cell number were still the same in untreated and treated normal cultures (Fig. 4A and B). The R-8₂-treated transformed cells, however, were drastically reduced in number, and the remaining cells were either vacuolated or had a normal morphology (Fig. 4D). DNA synthesis as measured by [³H]thymidine incorporation (26) and uptake of glucose as measured by

TABLE 2. [³H]Thymidine incorporation by normal and transformed cells treated with R-8₂

µg of drug/ml	% of control after 48 hr					
	0	3	5	10	15	20
N	100 (38,600)*	100	100	74	39	20
T	100 (44,600)	70	64	51	28	4

* Numbers in parentheses are dpm/mg of protein. Average of three experiments.

Procedure was as described in legend of Fig. 3 and Methods. N, normal cells; T, transformed cells.

2-[³H]deoxyglucose (26) showed the same pattern of preferential sensitivity to R-8₂ (Tables 2 and 3). The soluble pool of [³H]thymidine was not affected by the presence of R-8₂. The rate of protein synthesis as measured by [³H]leucine incorporation into the acid-insoluble pool was the least sensitive to the action of R-8₂, although transformed cells were still more affected (Table 3).

Effect of R-8₂ on Previously Formed Foci. To understand to what degree this preferential toxicity to transformed cells could explain the focus inhibition, two kinds of experiments were performed. In one series of focus assays, 10 µg/ml of R-8₂ was added 1 hr after infection, resulting in more than 90% inhibition of focus number after 8 days. If the action of R-8₂ was solely on inhibition of the initiation of transformation, one would expect that addition of R-8₂, 2 days later, would have no effect on the number of foci produced. In fact, when

TABLE 3. [³H]Leucine incorporation and [³H]deoxyglucose uptake (% of control after 48 hr) by normal and transformed cells treated with R-8₂

R-8 ₂	Leucine		2-Deoxyglucose	
	N	T	N	T
0	100 (32,100)*	100 (42,900)	100 (43,900)	100 (98,100)
3 µg/ml	100	100	108	72
5 µg/ml	100	88	105	53
10 µg/ml	85	—†	85	42

* The numbers in parentheses represent dpm/mg of protein.

† Too few cells left for determination. Average triplicate samples of one experiment.

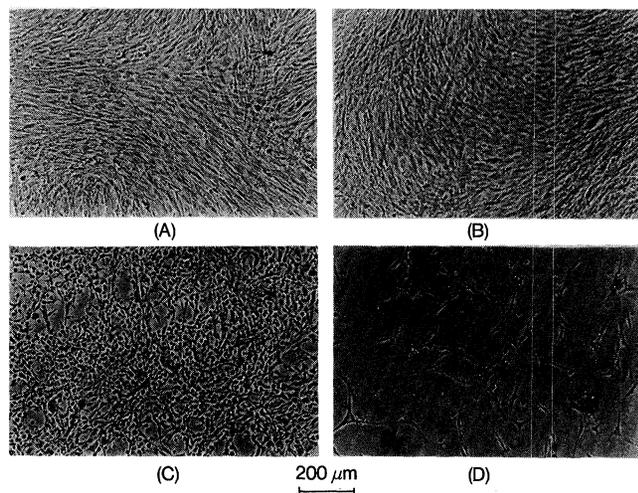


FIG. 4. Morphology of normal and transformed cells in the presence of R-8₂. Legend as in Fig. 3, except that cells were seeded at 1.5×10^4 cells per cm^2 and medium was changed on day 2. Control and treated ($5 \mu\text{g}/\text{ml}$ of R-8₂) cultures were photographed 3 days after seeding. (A and B) normal cells in the absence and presence of R-8₂. (C and D) transformed cells in the absence and presence of R-8₂.

R-8₂ was added 2 days after infection, there was still more than 40% inhibition of the foci if scored on day 8. Furthermore, the remaining foci were smaller than the control (compare Fig. 1C and D), and the percent inhibition decreased to about 10% by day 11 when the foci became quite visible. This experiment indicated to us that a large proportion of the focus inhibition observed (Table 1) may be attributed to the preferential toxicity of R-8₂ to transformed cells in addition to any inhibition of initiation of transformation.

In a second series of experiments, B-RSV foci were allowed to develop until they were visible (5 days). The agar was then removed gently and complete medium was added with or without $10 \mu\text{g}/\text{ml}$ of R-8₂. The foci continued to grow with a measurable rate in control cultures (Fig. 5A and B, Fig. 6). The removal of the agar resulted in a "necrotic" focus as the piled-up transformed cells in the center of the focus were lost to the medium (Fig. 5B). The growth of the foci after the addition of $10 \mu\text{g}/\text{ml}$ of R-8₂, however, was virtually arrested (Fig. 5C and D, Fig. 6). It has been shown previously that 48 hr after seeding, the normal chicken cells seeded at high density are no longer susceptible to transformation by B-RSV (22). The focus of B-RSV, therefore, is comprised essentially of the progenies derived from the initially transformed cell. Indeed, we observed no additional foci in control cultures, despite the fact that virus is released into the medium after agar removal. Thus, the lack of focus growth in the presence of R-8₂ is due to inhibition of transformed cell growth rather than an inhibition of secondary infection.

Mixed Cultures. Two experiments were performed in which 50% normal and 50% transformed cells were plated and allowed to grow in the presence and absence of R-8₂ ($5 \mu\text{g}/\text{ml}$) for 3 days. In the first experiment, where the cells were plated at a low density (1×10^4 cells per cm^2), cells grown in the absence of R-8₂ appeared to be completely transformed (Fig. 7A), while cultures grown in the presence of R-8₂ appeared to be largely normal by the end of the 3-day period (Fig. 7B). In the second experiment, where the cells were

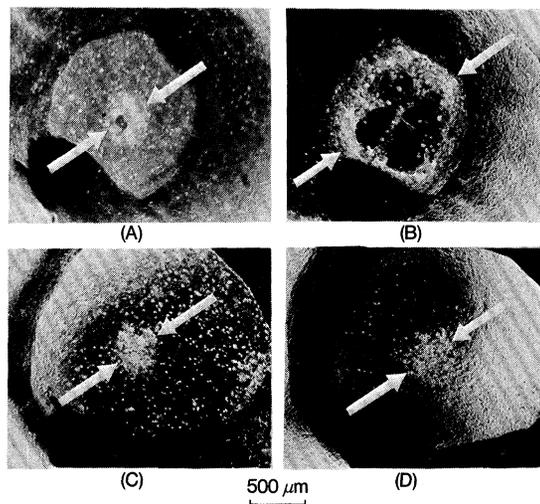


FIG. 5. Arrest of focus growth after addition of R-8₂. Two sets of focus assay plates of B-RSV were allowed to develop until foci were visible (5 days). The agar overlay was then removed and replaced with regular medium containing 0.1% (Me)₂SO or R-8₂ [$10 \mu\text{g}/\text{ml}$ in 0.1% (Me)₂SO]. Visible foci were encircled with black pen and numbered and they were then photographed on successive days. Arrows indicate the boundary of focus in each case. (A) control focus 1 hr after addition of liquid medium; (B) the same focus as in A, 82 hr later; (C) focus 1 hr after addition of liquid medium and R-8₂ ($10 \mu\text{g}/\text{ml}$); (D) the same focus as in C, 82 hr later.

plated at a higher density (5×10^4 cells per cm^2), cells grown without R-8₂ appeared to be completely transformed as expected. The cells with R-8₂ present, however, were still in a monolayer, and the majority of cells were normal, although dispersed transformed cells could be seen in the culture. Consistent with previously described results, these mixed culture experiments indicate a preferential inhibition and/or killing of transformed cells. Furthermore, they might suggest an additional role of R-8₂ in preventing secondary infection

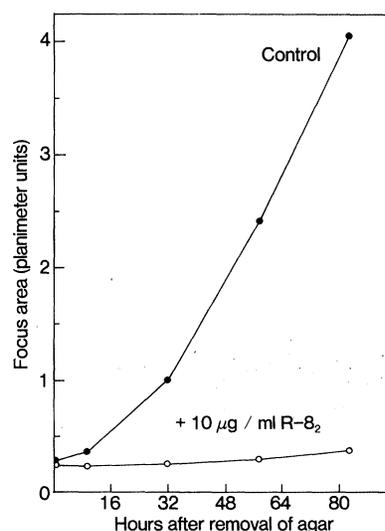


FIG. 6. The rate of growth of previously formed foci in the presence and absence of R-8₂. Experiment was performed as described in legend of Fig. 5. The area under the foci was estimated by use of a planimeter. Each curve is the average of three foci.

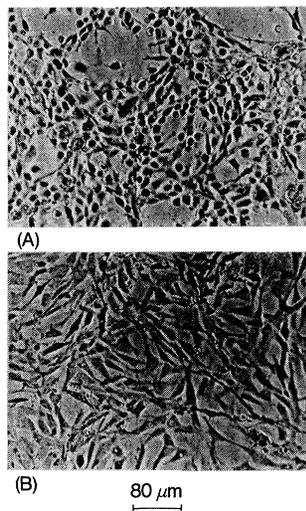


FIG. 7. Morphology of mixed cultures in the presence and absence of R-8₂. 50% normal and 50% transformed cells were seeded at a total population of 1×10^4 cells per cm². The pictures were taken 3 days after seeding. (A) culture with (Me)₂SO; (B) culture with R-8₂ (5 µg/ml).

by inactivating the virus itself. Whether the presence of R-8₂ in these experiments selects for normal cells by killing transformed cells, or whether it actually causes a reversion toward a normal morphology, is a question currently under investigation.

DISCUSSION

The selective inhibition and/or destruction of neoplastic cells while the normal cell growth and function continues, is a general aim of cancer chemotherapy. We have shown that rifazone-8₂, a specific inhibitor of RNA-directed DNA polymerase (21), also inhibits the growth of virus-transformed cells in tissue culture without appreciable side effects to normal cell growth. Preferential inhibition of transformed cells has been reported for rifampicin previously (18). However, rifampicin does not inhibit RNA-directed DNA polymerase appreciably (9), and the dosage needed for focus inhibition (more than 20 µg/ml) is toxic to normal chick cells in our hands.

There are a number of possible explanations for this increased toxicity of R-8₂ to the transformed cells. (1) There is evidence that cancer cells in general have altered permeability (27) and altered membrane properties (28), factors that could account for the observations described here. Once the nature of these differences is understood they may further be exploited to synthesize new derivatives that can cross the membrane of specific tumor cells more readily. (2) The drug may act partially by preventing transformation through inhibition of reverse transcriptase (12). If a continuing involvement of this enzyme in the growth of transformed cells is postulated, the additional inhibitory action of R-8₂ on the growth of transformed cells may be explained. (3) Alter-

natively, R-8₂ may be inhibiting an as yet unknown enzyme function(s) which might be essential to the growth of transformed cells. These possibilities are not mutually exclusive. The additional action of R-8₂ on the infectivity of the virus itself should also be investigated (12). To what extent a change in permeability of transformed cell membrane is responsible for the observed effects will be studied by use of radioactive derivatives.

The work described in this paper was sponsored, in part, by the U.S. Atomic Energy Commission and in part, by the National Cancer Institute, Grant NCI-1R0-1-CA14828-1. A.N.T. was supported by the Elsa U. Pardee Foundation for Cancer Research.

- Diggelmann, H. & Weissman, C. H. (1969) *Nature* **224**, 1277-1279.
- Richat, N. & Balduzzi, P. (1970) *Bacteriol. Proc.* **60**, 160.
- Calvin, M., Joss, U. R., Hackett, A. J. & Owens, R. B. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1441-1443.
- Hackett, A. J., Sylvester, S. S., Joss, U. R. & Calvin, M. (1972) *Proc. Nat. Acad. Sci. (USA)* **69**, 3653-3654.
- Hackett, A. J. & Sylvester, S. S. (1972) *Nature New Biol.* **239**, 166-167.
- Thompson, F. M., Tischler, A. N., Adams, J. & Calvin, M. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 107-109.
- Gallo, R. C., Yang, S. S. & Ting, R. C. (1970) *Nature* **228**, 927-929.
- Gurgo, C., Ray, R. K., Thiry, L. & Green, M. (1971) *Nature New Biol.* **229**, 111-114.
- Yang, S. S., Herrera, F., Smith, R., Reitz, M., Lancini, G., Ting, R. & Gallo, R. (1972) *J. Nat. Cancer Inst.* **49**, 7-25.
- Gurgo, C., Ray, R. & Green, M. (1972) *J. Nat. Cancer Inst.* **49**, 69-79.
- Green, M., Bragdon, J. & Rankim, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1294-1298.
- Wu, A. M., Ting, R. C. & Gallo, R. C. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1298-1302.
- Wu, A. M. & Gallo, R. C. (1974) *Biochim. Biophys. Acta*, in press.
- Smith, G. R., Whang-Peng, J., Gallo, R. C., Levine, P. & Ting, R. C. (1972) *Nature New Biol.* **236**, 166-171.
- Subak-Sharpe, J. H., Timbury, M. C. & Williams, J. F. (1969) *Nature* **222**, 341-345.
- Toolan, H. W. & Ledinko, N. (1972) *Nature New Biol.* **237**, 200-202.
- Robinson, H. L. & Robinson, W. J. (1971) *J. Nat. Cancer Inst.* **46**, 785-788.
- Vaheri, A. & Hanafusa, H. (1971) *Cancer Res.* **31**, 2032-2036.
- Medoff, G., Kawan, C. N., Schlessinger, D. & Kobayashi, G. S. (1973) *Cancer Res.* **33**, 1146-1149.
- Tischler, A. N., Joss, U. R., Thompson, F. M. & Calvin, M. (1972) *J. Med. Chem.* **16**, 1071-1075.
- Tischler, A. N., Thompson, F. M., Libertini, L. J. & Calvin, M. (1974) *J. Med. Chem.*, in press.
- Rubin, H. (1960) *Virology* **10**, 29-49.
- Rein, A. & Rubin, H. (1968) *Exp. Cell Res.* **49**, 666-678.
- Bissell, M. J., White, R. C., Hatie, C. & Bassham, J. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2951-2955.
- Dolberg, D. & Bissell, M. J. (1974) *In Vitro*, in press.
- Bissell, M. J., Hatie, C. & Rubin, H. (1972) *J. Nat. Cancer Inst.* **49**, 555-565.
- Bissell, M. J., Rubin, H. & Hatie, C. (1971) *Exp. Cell Res.* **68**, 404-410.
- Burger, M. M. (1971) "Cell surface in neoplastic transformation," in *Current Topics in Cellular Regulation*, eds. Horecker, B. L. & Stadtman, E. R. (Academic Press, New York), Vol. 3, pp. 135-193.