

Effect of Ascorbic Acid on Rhinovirus Replication in WI-38 Cells¹ (38724)

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Numerous field studies have been conducted in an effort to determine the efficacy of vitamin C treatment in preventing or alleviating symptoms of the common cold. The results have been equivocal mainly because the studies were usually though not always retrospective and depended on many subjective interpretations (1-5). Another and perhaps more objective approach to the problem is to investigate the effect of ascorbic acid on rhinovirus replication at the cellular level under conditions which can be controlled. To date, there have been limited attempts in this direction (6).

Preliminary tests in this laboratory suggest that ascorbic acid significantly inhibits rhinovirus replication in human diploid cells. Variables such as the concentration of ascorbic acid and its toxicity for human diploid cells, the thermal inactivation of rhinovirus in the presence of ascorbic acid, and the multiplicity of infection were considered in the design of experiments. Standardization and control of these variables was necessary before the effect of ascorbic acid on rhinovirus replication in diploid cells as demonstrated by one-step and multistep growth curves could be evaluated.

Materials and methods. Viruses. Rhinovirus, type 20 (RV20) was obtained from Dr. V. V. Hamparian, Ohio State University; Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) from Dr. T. Merigan, Stanford University. All virus stocks were stored at -70° .

Cell cultures. HeLa cells were given to us by Dr. Hamparian. WI-38 cells and human foreskin fibroblasts (HFF) were obtained through the courtesy of Drs. L. Hayflick and T. Merigan, respectively. Cells were grown in 12-oz and 4-oz prescription bottles

or 60-mm plastic petri dishes (Falcon Plastics, Los Angeles) depending upon need, such as virus production, interferon production, growth-curve studies, or plaque assays. Cultures were grown and maintained in Eagle's minimum essential medium (MEM) containing Earle's balanced salt solution, 10% fetal calf serum (FC), and antibiotics (200 units penicillin/ml, 50 μ g streptomycin/ml). Petri dish cultures were grown in a humidified, 4% CO₂ atmosphere at 36°. The WI-38 and HFF cell strains were used at the 21-25 and 16-20 population-doubling level ranges, respectively.

Solutions. A 1% stock solution of L-ascorbic acid (Schwarz/Mann, Orangeburg, NY) was prepared by dissolving its sodium salt in glass-distilled water. Reduced glutathione (Calbiochem, San Diego) was also prepared as a 1% stock solution in glass-distilled water. Both solutions were sterilized by Seitz filtration. Whenever ascorbic acid was added to growth medium for rhinovirus growth-curve experiments, glutathione was also added in equimolar proportions, unless specified otherwise, to maintain the ascorbic acid in the reduced form.

Rhinovirus plaque assay. The plaque assay employed is a modification of that described by Cooper (7) for assaying poliovirus infectivity. Barely confluent monolayers of HeLa cells in 60-mm petri dishes were inoculated with appropriate dilutions of rhinovirus preparations and incubated at 33.5° for 1 hr. The inoculum was then aspirated and the monolayer overlaid with 4 ml of MEM containing 10% FC and 0.45% agarose. After 4-day incubation at 33.5° in a humidified 4% CO₂ atmosphere, 3 ml of the above MEM-agarose overlay containing a 1/30,000 dilution of neutral red was added. Plaques were counted after an additional 24-hr incubation at 33.5°.

Interferon preparation. Rhinovirus growth-

¹ Supported in part by the Brown-Hazen Fund of the Research Corporation and by U. S. Public Health Service Training Grant AI-00082 from the National Institute of Allergy and Infectious Diseases.

curve samples to be tested for interferon activity were frozen at -20° , thawed, adjusted to pH 2.2 with 1 *N* HCl, kept at 4° for 24 hr, readjusted to pH 7.2 with 1 *N* NaOH, and centrifuged for 1-1/2 hr at 100,000g in a refrigerated Spinco 40.2 preparative rotor. The supernatant fluid was assayed for interferon immediately or within a few days after storage at 4° .

An interferon standard was prepared from confluent monolayer cultures of HFF cells in 12-oz prescription bottles infected with NDV at a multiplicity of 10 plaque-forming units (PFU)/cell. After overlaying with MEM + 10% FC and incubating for 24 hr at 36° the infected cultures were treated as above except that exposure to pH 2.2 at 4° was extended to 5 days before neutralization with 1 *N* NaOH and centrifugation for 1-1/2 hr at 100,000g. The supernatant fluid containing the interferon activity was ampulated and stored at -20° .

Interferon assay. Interferon activity was assayed by the 50% plaque reduction method similar to that described by Moehring *et al.* (8). Samples to be assayed were serially diluted at half-log intervals in MEM + 2% FC and 4-ml vol were left in contact with confluent, stationary 6-day-old cultures of HFF cells in 60-mm petri dishes for 24 hr at 36° . After aspirating and washing once with Hanks' balanced salt solution (HBSS), the cultures were inoculated with 40-80 PFU of VSV and incubated at 36° for 1 hr. The inocula were then aspirated and the cultures overlaid with 4 ml of MEM containing 10% FC and 0.45% agarose and incubated at 36° in a humidified 4% CO_2 incubator for 24 hr. Cultures were finally overlaid with 3 ml of the same agarose medium containing 1/30,000 dilution of neutral red, incubated overnight at 36° , and counted. Plates were routinely read at 48 hr postinfection. The dilution which reduced the plaque count by 50% was considered 1 unit of interferon, and the reciprocal of that dilution was the titer of the sample.

Growth curves. One-step and multistep growth curves of RV20 were carried out in WI-38 cells. Four-ounce prescription bottles were seeded with 1×10^5 cells in 10 ml of

MEM + 10% FC and incubated at 36° for at least 6 days at which time cultures were fully confluent. When growth of virus in ascorbic acid-treated cells was to be compared with that in control cultures, pretreatment of test cultures with growth medium containing appropriate concentrations of ascorbic acid plus glutathione or glutathione alone was carried out for 2 days before infection with RV20. For one-step growth curves, growth medium was removed from the cultures, 0.5 ml of virus was inoculated at a multiplicity of 6 PFU/cell, and the cultures were incubated at 36° for 1 hr. The inocula were aspirated and the cultures washed $2\times$ with 10 ml of HBSS before adding 10 ml of growth medium and incubating at 36° . Those cultures which had been pretreated with ascorbic acid plus glutathione or glutathione alone in growth medium were exposed to their respective reagents throughout the period of virus growth. Sample cultures were removed at appropriate intervals during a 17-hr growth period and then frozen and stored at -70° . They were frozen and thawed three times and clarified by centrifugation in a PR2 refrigerated centrifuge at 1500 rpm for 10 min before assaying. Multistep growth-curve experiments were carried out as above, except that cultures were infected at a multiplicity of 0.04 PFU/cell and sample cultures were harvested at intervals over a 48-hr growth period.

Results. Effect of ascorbic acid concentration on RV20 replication in WI-38 cells—a preliminary screening. Four-ounce bottle cultures of confluent WI-38 cells (6 days postseeding) were divided into two groups. The members of the test group were pretreated for 2 days with growth medium (MEM + 10% FC) containing various concentrations of ascorbic acid plus equimolar concentrations of glutathione. The control group received growth medium only. The cultures were inoculated with RV20 at a multiplicity of 0.04 PFU/cell and overlaid with their appropriate test or control medium and incubated at 36° for 2 days. Cultures were then frozen and thawed $3\times$ and assayed for infectivity. Relative total yields of infectivity from

ascorbic acid-treated and -untreated cultures are presented in Fig. 1. At the levels of 200 and 350 μg ascorbic acid/ml, the production of infective virus was inhibited approximately 80 and 90%, respectively, in the treated cells. Although 350 μg ascorbic acid/ml appeared not to be toxic to the WI-38 cells, a lower level of 250 $\mu\text{g}/\text{ml}$ was, nevertheless, selected for subsequent growth-curve experiments.

Effect of ascorbic acid on uninfected WI-38 cells. Preliminary experiments indicated that a concentration of ascorbic acid as low as 50 $\mu\text{g}/\text{ml}$ of growth medium was toxic for WI-38 cells during the log phase of growth. When the monolayer cultures became fully confluent (6 days postseeding), however, the cells could tolerate as much as 350 μg ascorbic acid/ml of growth medium for 4 days without any apparent toxic effect.

The viability of confluent uninfected WI-38 cells after a 4-day exposure to 250 μg ascorbic acid plus 437 μg (equimolar) glutathione per ml growth medium was assessed by determining the treated cells' capacity to be subcultured. Two 4-oz bottle cultures, grown to confluency in 6 days, were overlaid with growth medium containing

ascorbic acid plus glutathione and glutathione alone, respectively. A third culture received only growth medium. After 4 days' incubation at 36°, each culture was trypsinized and the cells counted and transferred to four 4-oz bottles at a seeding of 1×10^6 cells per bottle. These were then grown in growth medium without ascorbic acid and/or glutathione and cells counted at 6 and 8 days postseeding. The results of a typical experiment expressed as number of cells/subculture are summarized in Table I and indicate that ascorbic acid plus glutathione or glutathione alone had no adverse effect upon cell viability.

Effect of ascorbic acid on in vitro thermal inactivation of RV20 at 37°. A 10-fold dilution of stock RV20 (titer = 3×10^7 PFU/ml) was made in growth medium samples containing ascorbic acid, gluta-

TABLE I. GROWTH OF WI-38 CELLS AFTER EXPOSURE TO ASCORBIC ACID AND GLUTATHIONE FOR 4 DAYS.

Treatment ^a	No of cells/subculture after ^b	
	6 Days	8 Days
Growth medium + 250 μg ascorbic acid/ml + 437 μg glutathione/ml	3.2×10^6	5.0×10^6
Growth medium + 437 μg glutathione/ml	3.1×10^6	5.0×10^6
Growth medium alone	2.7×10^6	4.5×10^6

^a See text for experimental procedure.

^b Number of cells per subculture is an average value of two cultures in each case.

TABLE II. HALF-LIFE OF RV20 AT 37° IN PRESENCE OF ASCORBIC ACID AND GLUTATHIONE.

Diluent ^a	Half-life (hr)
Medium	1.0
Medium + glutathione	2.0
Medium + ascorbic acid	1.8
Medium + ascorbic acid + glutathione	2.4
Medium + 1/2 (ascorbic acid + glutathione)	2.0

^a Medium = MEM + 10% FC; glutathione = 437 $\mu\text{g}/\text{ml}$; ascorbic acid = 250 $\mu\text{g}/\text{ml}$.

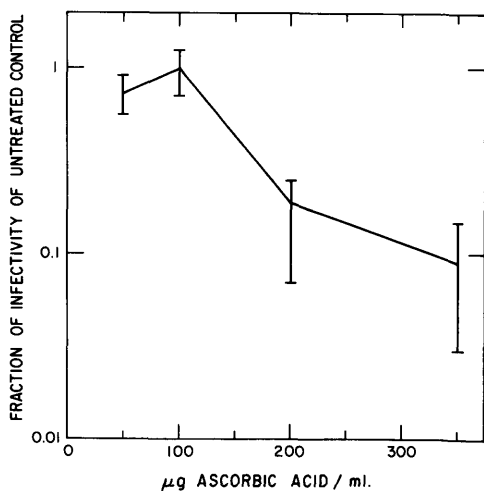


FIG. 1. Relative yields of RV20 infectivity from infected control and ascorbic acid-treated WI-38 cultures. Each point on the curve represents the average yield of virus from two to four ascorbic acid-treated cultures relative to the yield from untreated control cultures. The vertical bars indicate the range for each point. See text for experimental details.

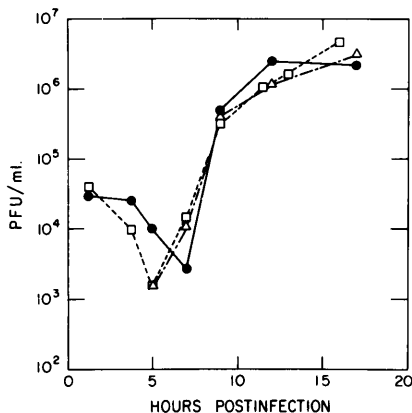


FIG. 2. One-step growth curves of RV20 in WI-38 cells. Cultures were infected at a multiplicity of 6 PFU/cell and incubated at 36°. The media used to pretreat cultures for 2 days prior to infection and during virus growth were: growth medium (MEM + 10% FC) (\square), growth medium plus 437 μ g glutathione/ml (Δ), and growth medium plus 437 μ g glutathione/ml and 250 μ g ascorbic acid/ml (\bullet).

thione, and mixtures of both. Each sample was divided into 1.0-ml aliquots in stoppered tubes and incubated in a 37° water bath. At appropriate time intervals samples of each were removed and stored frozen at -20° until assayed for surviving infectivity. Inactivation in all cases was exponential over a 24-hr period. The half-life values are summarized in Table II. Ascorbic acid and glutathione had a sparing effect on virus infectivity as might be expected of reducing agents.

One-step growth of RV20 in WI-38 cells in the presence of ascorbic acid. The growth of RV20 in confluent monolayer WI-38 cultures infected at a multiplicity of 6 PFU/cell was determined in growth medium containing ascorbic acid plus glutathione, glutathione alone, or neither reagent as described in Materials and Methods. The results are plotted in Fig. 2. The pattern of one-cycle growth of RV20 appears to be the same in the absence or presence of ascorbic acid and glutathione with the possible exception of a slight delay in the initiation of virus replication in the presence of the mixture of both of these reagents. The kinetics of RV20 one-cycle growth in monolayer cultures resemble that reported for several rhinovirus serotypes by others (9, 10),

namely, an eclipse period of 5–7 hr with peak virus production from 10–15 hr postinfection.

Multistep growth of RV20 in WI-38 cells in the presence of ascorbic acid. The procedure was the same as that for the one-step growth curve experiment, except that the WI-38 cultures were infected at a multiplicity of 0.04 PFU/cell. The initial rates of virus replication during the first cycle of growth were similar under all conditions of growth, i.e., in the absence or the presence of ascorbic acid, glutathione, or a mixture of both, up to 16 hr postinfection. Thereafter, the virus yield increased at progressively slower rates in the absence or presence of ascorbic acid or glutathione alone leveling off at 32 hr. In the presence of the mixture of ascorbic acid plus glutathione, however, the virus yield dropped gradually from 16–48 hr postinfection to 1/20 at 32 hr and 1/40 at 48 hr of that of the controls (Fig. 3).

Effect of multiplicity of infection (m.o.i.) upon the relative yields of RV20 in ascorbic acid-treated and -untreated WI-38 cells. Six-day-old confluent cultures of WI-38 were pretreated with a mixture of glutathione

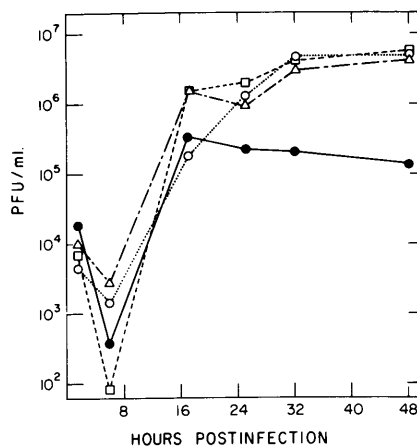


FIG. 3. Multistep growth curves of RV20 in WI-38 cells. Cultures were infected at a multiplicity of 0.04 PFU/cell and incubated at 36°. The media used to pretreat cultures for 2 days prior to infection and during virus growth were: growth medium (MEM + 10% FC) (\square), growth medium plus 437 μ g glutathione/ml (Δ), growth medium plus 250 μ g ascorbic acid/ml (\circ), and growth medium plus 437 μ g glutathione/ml and 250 μ g ascorbic acid/ml (\bullet).

plus ascorbic acid as described for growth-curve experiments in Materials and Methods. They were then inoculated over a range of multiplicities of $4-4 \times 10^{-5}$ PFU/cell in 10-fold dilution intervals and incubated at 36° in growth medium containing ascorbic acid plus glutathione. Sample cultures were removed for infectivity assays at 16 and 48 hr. Infected cultures untreated and treated with glutathione alone were included as controls. The results as summarized in Fig. 4 show that at 16 hr the yields of infective virus are roughly proportional to the m.o.i. and similar in all three sets of conditions, i.e., untreated or treated with ascorbic acid plus glutathione or glutathione alone. At 48 hr postinfection, however, the yield of infective virus in cells exposed to ascorbic acid plus glutathione was 1-1.5 log units less than that of glutathione-treated and -untreated control cultures at all levels of infection with the exception of the lowest m.o.i., namely, 4×10^{-5} PFU/cell.

The data have been assembled as a ratio of 48 hr to 16 hr yields of virus and plotted against m.o.i. in Fig. 5. Ratios less than 1 suggest that the virus replicated for one cycle during the first 16 hr of infection and thereafter suffered loss of infectivity without replacement by additional cycles of replication during the rest of the 48-hr period.

Assay of growth curve samples for interferon. Growth-curve samples were prepared for interferon assay and assayed by the plaque reduction method as described in Materials and Methods. No interferon activity was detected in multistep growth-curve samples of ascorbic acid treated or untreated cultures which had been infected at a multiplicity of 0.04 PFU/cell. In one single-step growth experiment (m.o.i. = 6), however, an interferon-like activity was present at low levels in samples taken at 5-17 hr postinfection from ascorbic acid-plus glutathione-treated cultures but not from untreated and glutathione-treated control cultures. Results are presented in Table III.

Attempts to improve interferon production by changing single-step growth curve conditions, e.g., by lowering serum concentration in growth medium from 10% to 2% and by increasing the *in vitro* age of WI-38 cell cultures from 6 to 16 days (11), were not successful. In fact, in all single-step growth curves tested other than the one summarized in Table III, the only suggestion that interferon was produced in ascorbic acid-plus glutathione-treated WI-38 cells was a reduction in plaque size and a slight, variable reduction in plaque number at 17 hr postinfection when growth-curve samples were

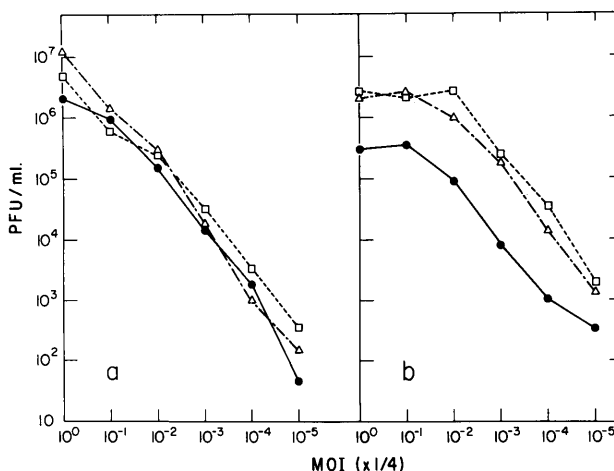


FIG. 4. Effect of multiplicity of infection (m.o.i.) on RV20 production in WI-38 cells. Sample cultures were withdrawn for infectivity assay at (a) 16 hr postinfection and (b) 48 hr postinfection. The media used to pre-treat cultures for 2 days prior to infection and during virus growth were: growth medium (MEM + 10% FC) (□), growth medium plus 437 μ g glutathione/ml (Δ), and growth medium plus 437 μ g glutathione/ml and 250 μ g ascorbic acid/ml (●).

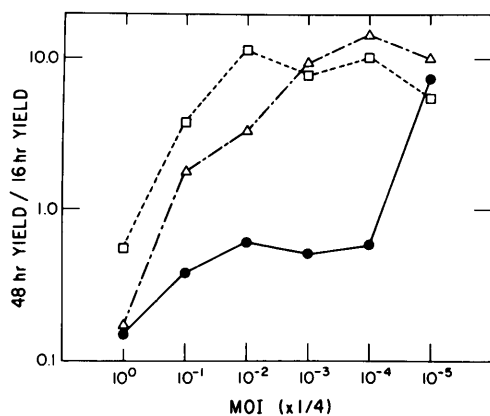


FIG. 5. Effect of multiplicity of infection on the ratio of 48 hr/16 hr yields of infectivity from RV20-infected WI-38 cells. Cultures were infected and incubated as described in Fig. 4. Media used were: growth medium (MEM + 10% FC) (□), growth medium plus 437 μ g glutathione/ml (Δ), and growth medium plus 437 μ g glutathione/ml and 250 μ g ascorbic acid/ml (\bullet).

TABLE III. INFECTIVITY AND INTERFERON ACTIVITY PRODUCED DURING ONE CYCLE OF GROWTH OF RV20 IN WI-38 CELLS^a IN PRESENCE OF ASCORBIC ACID PLUS GLUTATHIONE^b

Sample (hr postinfection)	Pfu/ml before preparation for interferon assay	Interferon titer ^c
5.0	1.0×10^4	22
7.0	2.8×10^3	15
9.0	5.0×10^5	42
12.0	2.5×10^6	0
17.0	2.2×10^6	46
Positive control ^d	—	480

^a M.o.i. = 6 PFU/cell.

^b Ascorbic acid = 250 μ g/ml; glutathione = 437 μ g/ml.

^c See Materials and Methods.

^d Positive control = a standard interferon produced in HFF cells infected with NDV and prepared as described in Materials and Methods.

assayed by the plaque-reduction method using the VSV-HFF cell system.

A preliminary investigation of the response of RV20 to the action of interferon in WI-38 cells revealed this rhinovirus serotype to be fairly sensitive. A standard interferon produced in NDV-infected HFF cells as described in Materials and Methods was tested for its capacity to reduce rhinovirus

plaque number in WI-38 cells. RV20 responded to 10 units of interferon with 99% reduction in plaque number, to 5 units with a 65% reduction, and to 0.5 units with a 20% reduction. While a direct comparison with VSV in WI-38 cells was not made, RV20 seems to have the same order of sensitivity to interferon in WI-38 cells as VSV has in HFF cells.

Discussion. The results of our experiments show that a mixture of ascorbic acid and glutathione does not adversely affect the production of RV20 in WI-38 cells at 36° during the first cycle of growth (Fig. 2). Subsequent cycles of virus replication, however, appear to be partially suppressed with yields at 48 hr postinfection from ascorbic acid- plus glutathione-treated cells equaling 1/30–1/40 of that from control cells (Fig. 3). This occurs at a concentration of ascorbic acid plus glutathione which does not affect the viability of WI-38 cells (Table I) and which enhances, to some extent, the thermal stability of extracellular virus (Table II).

Suppression of multicyclic replication of RV20 was observed over a wide range of multiplicities from 4×10^{-1} to 4×10^{-4} PFU/cell, i.e., the 48-hr/16-hr yield ratios were <1 (Fig. 5). These findings suggest that, in the presence of the ascorbic acid and glutathione mixture, an inhibitor of virus replication is produced in and released from infected cells during the first cycle of virus growth which inhibits subsequent cycles of virus replication. At a m.o.i. of 4 PFU/cell and higher, virus replication is not suppressed since most if not all cells are initially infected and only one cycle of growth is possible (Fig. 2). At the extremely low m.o.i. of 4×10^{-5} PFU/cell, however, the putative suppressor substance produced during the first cycle of growth may be too dilute to inhibit later multicyclic growth and thus may account for the observed 48-hr/16-hr yield ratio >1 .

A possible candidate for this inhibiting role is interferon. When samples drawn from both one-step and multistep growth experiments in the presence of ascorbic acid plus glutathione were prepared and assayed for interferon, only those of a one-cycle growth experiment were found measurably

active albeit at a low level (Table III). All controls, i.e., growth experiments carried out in growth medium alone or in growth medium containing only glutathione, yielded no demonstrable interferon activity.

Rhinoviruses are generally conceded to be poor inducers of interferon and also to be relatively unresponsive to its action, at least *in vitro*. Fiala (11) found that RV2 produced only minimal amounts of interferon in human embryonic kidney cells infected at a m.o.i. of 30 and noted that it responded to the effects of a standard interferon with 1/3 to 1/40 the sensitivity of VSV in terms of reduced virus yields in these same cells. Similarly, Stoker *et al.* (12) found that RV13, RV14, and RV20 induced no detectable interferon production in HeLa cells although these strains did respond to interferon with reduced yields of virus in HeLa cells. RV20 was the most sensitive of the three strains approaching VSV in its responsiveness. This is compatible with our finding that RV20 grown in WI-38 cell cultures in the presence of ascorbic acid plus glutathione does induce interferon production at barely detectable levels to which it appears sufficiently sensitive to suffer partial suppression of multicyclic growth. However, whether the ascorbic acid plus glutathione in some way potentiates the production of interferon in RV20-infected WI-38 cells, increases the sensitivity of the virus to inhibition by interferon, or mediates the inhibition of virus growth by an unrelated mechanism not involving interferon is not yet clearly resolved.

Summary. Exposure of WI-38 cells to ascorbic acid plus glutathione mixtures in

growth medium for 2 days prior to infection with rhinovirus serotype 20 and during virus replication suppressed multicyclic but not single-cycle growth of the virus. Multicyclic growth was suppressed over the range of multiplicity of infection (m.o.i.) of 4×10^{-1} – 4×10^{-4} PFU/cell. There was some suggestion that, in the presence of ascorbic acid plus glutathione, interferon was produced at the highest m.o.i. tested but at barely detectable levels.

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Received Oct. 7, 1974. P.S.E.B.M., 1975, Vol. 148.