

## **Ascorbic acid inhibits replication and infectivity of avian RNA tumor virus**

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## Abstract

Ascorbic acid, at nontoxic concentrations, causes a substantial reduction in the ability of avian tumor viruses to replicate in both primary avian tendon cells and chicken embryo fibroblasts. The virus-infected cultures appear to be less transformed in the presence of ascorbic acid by the criteria of morphology, reduced glucose uptake, and increased collagen synthesis. The vitamin does not act by altering the susceptibility of the cells to initial infection and transformation, but instead appears to interfere with the spread of infection through a reduction in virus replication and virus infectivity. The effect is reversible and requires the continuous presence of the vitamin in the culture medium.

The mechanism of action of ascorbic acid, especially in relation to its effect on viruses, remains unknown at a cellular and molecular level (1) despite the widespread popular belief in its various beneficial roles in combating the common cold and cancer (2, 3). While studying the effect of this compound on collagen synthesis in primary avian tendon (PAT) cells (4), we observed that transformation of these cells by Rous sarcoma viruses (RSV) proceeded at variable and unpredictable rates. We discovered that the observed variability was due to the presence or absence of ascorbic acid in the culture medium. Furthermore, pretreatment of cells with ascorbic acid before viral infection made the cultures more resistant to the spread of virus infection. We therefore set out to examine in more detail the effects of this vitamin on the ability of avian RNA tumor viruses to infect and transform cells in culture.

## Materials and Methods

### CELL CULTURE AND VIRUS INFECTION

PAT cells and chicken embryo fibroblasts (CEF) were prepared from 16- and 10-day-old chicken embryos, respectively, as described (4, 5). Cells were infected with various strains of Rous sarcoma viruses (6, 7), including a temperature-sensitive mutant virus, LA-24 (Prague, Subgroup A), with a defect in the src gene (8). Focus-forming units and infectious centers were determined according to Rubin (9).

### BIOCHEMICAL TECHNIQUES

Ascorbic acid levels were determined by using the reduction of ferric to ferrous ion by ascorbic acid and the ability of  $\alpha,\alpha'$ -dipyridyl to complex the ferrous form (10). Techniques for measuring 2-deoxy-D-[ $^3\text{H}$ ]glucose uptake and the rate of collagen synthesis have been described (4, 5). Reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) activity in the medium was measured as described (11, 12).

### MATERIALS

Tissue culture materials were from GIBCO. Radioactive materials were from New England Nuclear. Ascorbic acid was from Baker and of analytic reagent quality. Sodium isoascorbate was from K & K.

## Results

Ascorbic acid is very unstable under the usual culture conditions (13, 14). We determined that its half-life in our culture medium at 39°C (with or without cells) was only 1 hr (Fig. 1). A preliminary dose-response curve indicated that ascorbic acid at final concentrations greater than 10 µg/ml of medium (60 µM) was maximally effective in the variables discussed below provided that the medium was changed often (every 12 hr). Because there was no toxicity to these cells even at 75 µg/ml (see below), a final regimen of 50 µg/ml (0.28 mM) and daily changes of medium (every 24 hr) was adapted throughout unless otherwise indicated. The morphology of infected PAT cells was distinctly different depending on whether or not the cells were pretreated and grown in medium containing ascorbic acid (Fig. 2). The untreated cultures became entirely transformed after 5-6 days, as shown by their characteristic morphology (Fig. 2 Upper right) and infectious center assays (data not shown; ref. 9); the ascorbic acid-treated cells showed discrete foci of transformation which usually did not spread to the rest of the culture (Fig. 2 Lower right). Similar differences were obtained with other subgroups of the virus, although the exact morphological patterns varied depending on the subgroup of virus used.

To determine whether other criteria of transformation (in addition to morphology) were affected, uptake of 2-deoxyglucose was measured in normal and virus-transformed cells grown in the presence or absence of ascorbic acid. An increase in glucose uptake after RNA tumor virus infection is an accepted criterion of malignant transformation (see, for example, ref. 15). Although the rate of sugar uptake varies with the population density, the rate of growth, and the cell shape (5, 16), it is still higher in virus-transformed cells when these factors are controlled (5, 17). A comparison of 2-deoxyglucose uptake virus-infected cells (Fig. 3) indicated that cells pretreated with and grown in the presence of ascorbic acid had a greatly reduced rate of sugar transport. However, there was also a consistent decrease in the rate of sugar transport in both normal cells and cells infected with a transformation-defective virus after ascorbic acid treatment (Fig. 3B). Nevertheless, the magnitude of the change in glucose transport in normal cells was usually less than that observed in ascorbic acid-treated, transformed cells.

Because glucose uptake was also decreased in ascorbic acid-treated normal cultures, it was important to measure another biochemical transformation parameter. A decrease in collagen synthesis accompanies viral transformation in both CEF (18) and PAT cells (6). Whereas the level of collagen synthesis in transformed cells is not modulated by ascorbic acid (6), the rate of collagen synthesis in pretreated cells that were infected with RSV and kept in the presence of ascorbic acid was intermediate between the normal and transformed cells both in terms of absolute values and in terms of percent collagen in relation to other proteins: 3.8% in normal cells; 2.4% in normal cells in the presence of ascorbic acid; 1.5% in cells transformed by Prague A RSV; 1.7% in transformed cells when ascorbic acid was added after transformation; and 4.1% in cells transformed in the presence of ascorbic acid (data not shown further). Thus, in addition to relatively normal morphology, cultures infected with transforming viruses of RSV and grown

in ascorbic acid were more "normal" in that they had a depressed rate of sugar uptake and an increased rate of collagen synthesis relative to untreated controls.

Additionally, viral replication and infectivity, as measured by focus-forming units, was reduced in the presence of ascorbic acid (Table 1). The degree of inhibition varied depending on the culture conditions, the length of infection, and the subgroup of the virus. Nevertheless, when virus production was compared on a per cell or mg of protein basis, there was a reduction to at least 1/3rd and at times to 1/100th the original number when ascorbic acid was present. Total production of virus particles [as measured by reverse transcriptase assay (11, 12)] was also reduced, but the inhibition was slightly less than that of focus-forming units. Cellular transformation was not necessary for a decrease in virus number because transformation-defective viruses (8) also showed a substantial and comparable decrease in virus replication as measured by reverse transcriptase assay (Table 1).

Hydrogen peroxide formed as a result of cellular oxidation of ascorbic acid causes cytotoxicity to several different cell types, including CEF (19, 20). Under our culture conditions there was no toxicity to either normal or transformed cells at 50  $\mu\text{g}$  of ascorbic acid per ml when cell number was measured over a 5-day period (Fig. 4). Therefore, preferential toxicity to transformed cells could not explain the above findings. At levels above 100  $\mu\text{g}/\text{ml}$ , ascorbic acid showed some toxicity to both normal and transformed cells, especially in CEF cultures.

Three possibilities for the mechanism of action of ascorbic acid were considered: (i) Ascorbic acid could directly inactivate the virus (both the initial inoculum and the replicated virus) to reduce primary and secondary infection; (ii) pretreatment with ascorbic acid could render the cells resistant to viral infection by an unknown mechanism (e.g., by increasing collagen synthesis which, in turn, could form a "protective matrix" and prevent infection); and (iii) ascorbic acid could interfere with viral replication and infectivity by altering the metabolic makeup of the cell and by interfering with some of the steps in viral assembly and release. Ascorbic acid inactivates double and single-stranded RNA and DNA bacteriophages (21, 22). However, pretreatment of the different subgroups of RNA tumor viruses with ascorbic acid (50-500  $\mu\text{g}/\text{ml}$  for 1-4 hr) did not reduce their infectivity in subsequent focus assays. Indeed, there was a small but reproducible increase in focus-forming units when virus was preincubated with ascorbic acid (data not shown). Although the reason for this small increase in focus-forming units is not known, the first possibility was ruled out. The second and third possibilities were difficult to distinguish experimentally. Nevertheless, we favor the third possibility, based on the following four lines of evidence.

(i) In experiments where the initial level of infection was varied by changing the virus inoculum by a factor of 100, the degree of inhibition of virus production by ascorbic acid was independent of the multiplicity of infection (Table 2).

(ii) If the only effect of ascorbic acid was to prevent the initial virus infection, the fully infected cultures should be unaffected by the presence or the absence of the vitamin. Yet, removal of ascorbic acid from the culture medium reversed the inhibition of virus titer and its addition, even after transformation, reduced the yield of infectious virus (Table 3). Whether or not the observed effects of ascorbic acid on cellular transformation were also reversible after infection could not

be demonstrated clearly. Experiments with temperature-sensitive mutants of RSV (LA24), where the cells were infected at the nonpermissive temperature and shifted to the permissive temperature in the presence or the absence of ascorbic acid, gave variable results when 2-deoxyglucose transport was measured. Nevertheless, virus titers were always decreased when shifted in the presence of ascorbic acid and increased when ascorbic acid was removed (data not shown).

(iii) In addition to the well-known effects of ascorbic acid on collagen synthesis, the data presented in Fig. 3 indicated that addition of ascorbic acid to untransformed cells also led to a consistent reduction in the rate of sugar transport. This inhibition was not due to competition for the glucose-transport protein (1) because ascorbic acid was not present during the uptake studies. There were other changes in the metabolite patterns of the cells, including a reduction in lactic acid production, as might be expected from the lowered sugar uptake (ref. 17 and unpublished observations). Thus, ascorbic acid is affecting additional host cell processes which, in turn, may be responsible for inhibition of viral replication.

(iv) Most importantly, if monolayers were prepared from ascorbic acid-pretreated normal cells and were infected with virus that was grown in the absence of ascorbic acid, no reduction in the number of foci were observed, although the average size of a focus was smaller in ascorbic acid-treated cultures (Table 4).

To determine whether or not compounds with the same reducing potential as ascorbic acid but different biological activity *in vivo* could substitute for the vitamin, we used its epimer, D-isoascorbic acid, which is reported to have 1/20th of the biological activity of ascorbic acid (23). At 0.28 mM, isoascorbic acid had effects very similar to those of ascorbic acid in reducing the virus titer, decreasing the rate of uptake of 2-deoxyglucose, and increasing the rate of collagen synthesis although the magnitude of inhibition was more variable from experiment to experiment. At concentrations below 0.1 mM, however, isoascorbic acid was less effective, especially in decreasing 2-deoxyglucose transport. We did not measure the stability of isoascorbic acid under our culture conditions; thus, the small differences in the potency of ascorbic acid and isoascorbic acid may be related to their differential stability or differential purity.

## Discussion

The following picture has emerged from our results: Ascorbic acid at concentrations that are not toxic to either normal or virus-infected cells (0.06-0.3 mM) causes a reduction in virus replication and infectivity in both PAT and CEF cultures infected with RNA tumor viruses to 1/3rd to 1/100th the normal number of particles. The effect is observed after a 15- to 20-hr treatment of the culture with the vitamin and is dependent on its continuous presence. The effect is not due to interference with virus attachment, penetration, infection, integration, and transformation, but to interference with the production of virus particles. Furthermore, the virus produced in the presence of ascorbic acid may not be as infectious as the virus produced in the absence of ascorbic acid

The mechanism by which ascorbic acid brings about the reduction in virus titer and infectivity is not clear. The inhibition of virus replication occurs in both PAT and CEF cultures despite the fact that the former produces a 10- to 15-fold higher level of collagen (4) than the latter. Additionally, as shown in Table 4, the initial infection is not affected by pretreatment of cultured cells with ascorbic acid. Therefore, increased collagen production brought about by the presence of ascorbic acid is not involved directly in reduction of virus titers. Studies with isoascorbic acid indicate that the results reported here may be due to the reducing potential of these compounds. Blank and Peterkofsky (24) have shown that ascorbic acid could be concentrated 10-fold by CEF and that the H2O2 produced either internally or at the cell surface is responsible for the toxicity observed in these cultures at low density (19). It is possible that the naked virus may be sensitive to the intracellular ascorbic acid or its oxidation products just as are bacterial phages (21, 22). Once the virus is packaged and released, however, it does not seem to lose activity by ascorbic acid treatment.

Two additional points merit discussion. Despite the fact that virus titer and infectivity are substantially lower in ascorbic acid-treated cultures, the cells still produce an appreciable amount of infectious virus (Tables 1-3). Yet, regardless of how long the virus-infected, ascorbic acid-treated cultures are maintained, some cells continue to look flat and appear normal (Fig. 2). There are two possible reasons for this latter observation. The first is the possibility of interferon induction. RSV induces interferon in chicken cells (25). Additionally, ascorbic acid increases interferon production in mouse embryo fibroblasts by poly(rI)-poly(rC) and in human embryo lung fibroblasts infected with Newcastle disease virus (26, 27). The latter results, however, have not been confirmed in other laboratories. WI-38 cells treated with ascorbic acid and glutathione and infected with rhinovirus did not show much interferon production (28). Nevertheless, it is conceivable that ascorbic acid, under our culture conditions, could increase interferon production by RSV-infected cultures and hence further retard virus spread. The other likely possibility is that some of the cells that appear normal may, indeed, be infected. Because ascorbic acid causes the production of some defective virus (as shown by the fact that the production of focus-forming units is inhibited more severely than reverse transcriptase activity), cells could be infected by the transformation-defective subpopulation, hence becoming resistant to superinfection by the transforming virus (8).

Schwerdt and Schwerdt (28) have shown that ascorbic acid reduced rhinovirus replication in cultured human cells after the first cycle of virus replication. The "delayed" action of ascorbic acid on rhinovirus replication is reminiscent of its action on RSV replication in chicken cells. These authors, however, interpret their data solely on the basis of interferon production, a possibility that is almost ruled out by their own data. It is more likely that in their system also the presence of ascorbic acid caused defective or reduced virus replication by a mechanism other than by elaboration of interferon. Their data differ from ours in that the amount of ascorbic acid was 250  $\mu$ g/ml of medium and had to exceed 100  $\mu$ g/ml before any effect on virus replication could be observed. The results reported here were obtained with 10-50  $\mu$ g of ascorbic acid per ml and were independent of culture density. Ascorbic acid in excess of 100  $\mu$ g/ml was toxic to CEF under our conditions, and others have reported toxicity at lower concentrations (19, 20). Furthermore, at a multiplicity of infection greater than 4 plaque-forming units per cell, rhinovirus replication was not suppressed by ascorbic acid in WI-38 cells. We have shown here that in

chicken cells infected with retroviruses, there is a substantial decrease in virus replication after addition of ascorbic acid, regardless of the initial multiplicity of infection. Whether these differences are species specific or whether they reflect differences between cell lines and primary cultures or differences in methods of procedure remains to be established .

It is important to emphasize that ascorbic acid at high concentration has been reported to have undesirable side effects, such as enhancement of chromosome damage (29) and production of increased amounts of possible carcinogens (30). Much needs to be learned about the mechanism(s) of action of this simple, yet amazingly versatile, compound before clear conclusions can be drawn. The significance of our finding with regard to the action of ascorbic acid on viral infection in other species and, specifically, with regard to the common cold or cancer in humans remains obscure.

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## Figures and Tables

FIGURE 1

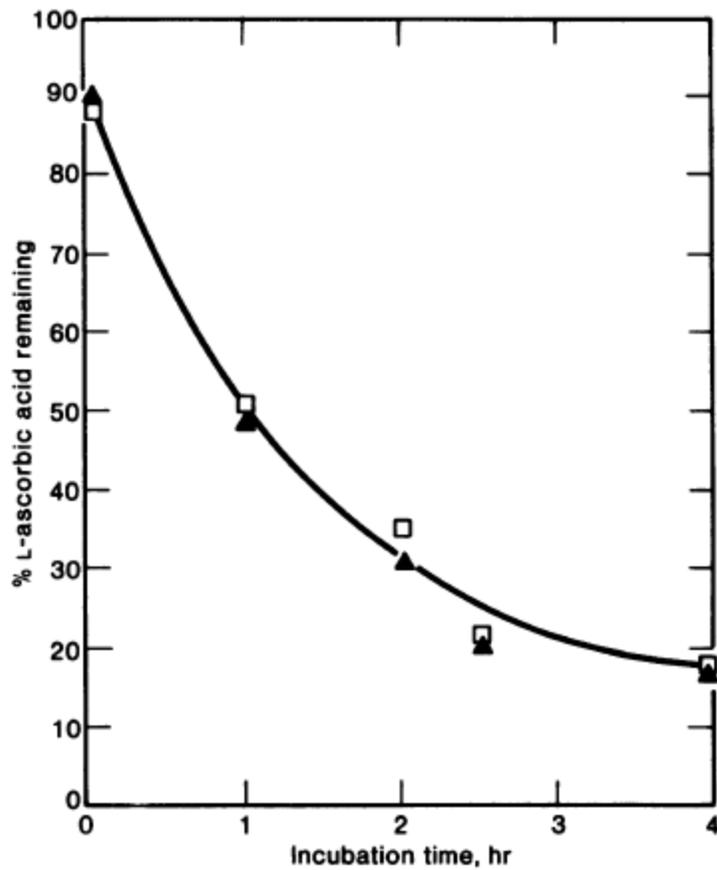
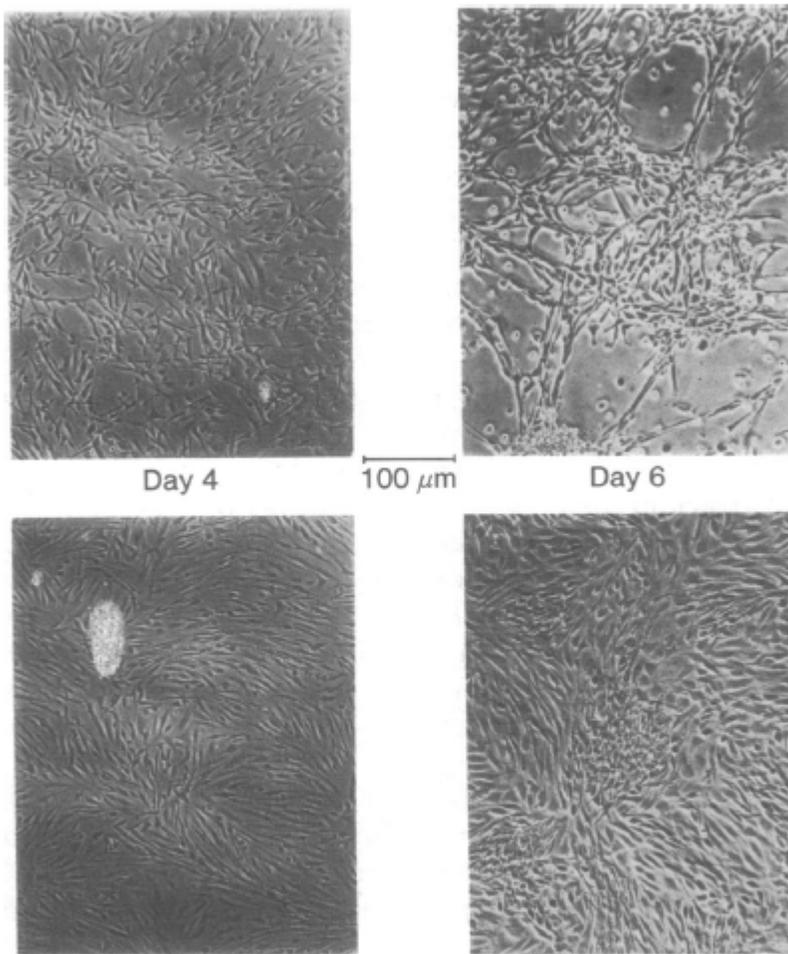


FIG. 1. Rate of disappearance of ascorbic acid from culture medium. PAT cells were seeded at  $0.5 \times 10^6$  (▲) or  $1.2 \times 10^6$  (□) cells per 25-cm<sup>2</sup> flasks and grown at 38°C as described (5). At indicated times, medium was removed and assayed for ascorbic acid by using  $\alpha, \alpha'$ -dipyridyl and ferric ion (10).

FIGURE 2



**FIG. 2. Morphology of ascorbic acid-treated PAT cells. Cells were prepared and infected with RSV (Prague, subgroup A). (Lower) Cultures received medium containing ascorbic acid (50 μg/ml) 24 hr prior to infection and in subsequent daily changes of medium. (Upper) Cultures without ascorbic acid. Pictures were taken 4 and 6 days after infection.**

FIGURE 3

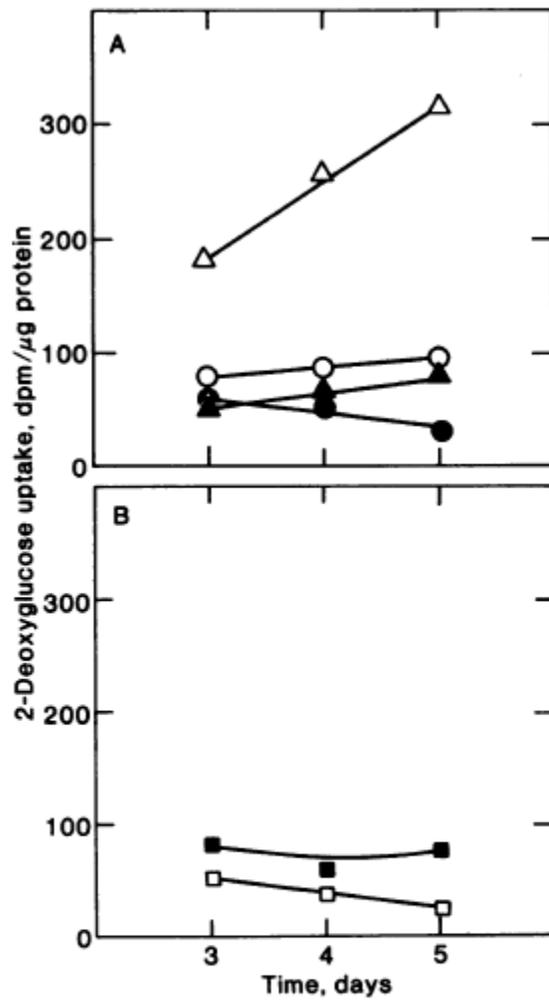


FIG. 3. Uptake of 2-[<sup>3</sup>H]deoxyglucose in normal and virus-infected cells treated with ascorbic acid. Cells were grown as in the legend for Fig. 2 and infected with either (A) Prague C strain or (B) a transformation-defective Prague C strain. No ascorbic acid: ▲, normal cells; △, cells infected with Prague C strain; ■, cells infected with transformation-defective Prague C strain. With ascorbic acid: ●, normal cells; ○, cells infected with Prague C strain; □, cells infected with transformation-defective Prague C strain.

FIGURE 4

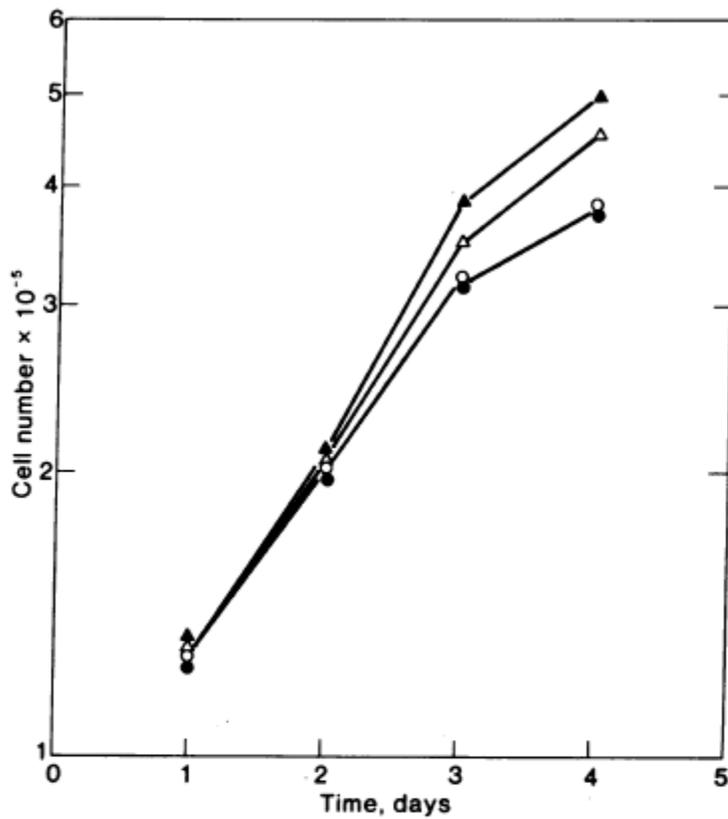


FIG. 4. Growth rate of PAT cells with and without ascorbic acid. Legend as for Fig. 2 except that the cells were infected with Prague C RSV. The higher density in ascorbic acid-treated Prague C cultures reflects the fact that transformed cells in the absence of ascorbic acid come off the dish, especially at higher densities. In the presence of ascorbic acid, the "normal" cells (see Fig. 2) form a background "matrix" and the transformed cells are retained on the top. PAT cells: ▲, with ascorbic acid; △, without ascorbic acid. ● and ○, Normal cells with and without ascorbic acid.

TABLE 1

Table 1. Ascorbic acid inhibition of virus replication and infectivity in PAT cells

Culture	Day 3		Day 4		Day 5	
	RT	FFU	RT	FFU	RT	FFU
Prague C	28,760	$3.2 \times 10^5$	61,330	$6.2 \times 10^5$	127,660	$1.4 \times 10^6$
Prague C + ascorbate	9,380	$2.5 \times 10^3$	22,870	$0.7 \times 10^5$	23,790	$1.8 \times 10^5$
% Inhibition	67	>99	63	88	81	87
Transformation-defective Prague C	18,580	—	14,870	—	23,850	—
Transformation-defective Prague C + ascorbate	5,740	—	6,640	—	1,400	—
% Inhibition	69	—	55	—	84	—

Cells were grown and infected as in the legend for Fig. 2. Medium was removed and 1 ml of appropriate medium was added and collected 2 hr later. The result is the average of duplicate plates of two experiments. Other procedures were as in the legend for Fig. 3. RT, reverse transcriptase activity: cpm per ml of medium, measured as described (11). FFU, focus-forming units per ml of medium, measured as described (9).

TABLE 2

Table 2. Lack of effect of initial virus inoculum on inhibition of infectious virus particles by ascorbic acid

Culture	FFU/mg of protein*		
	Day 2	Day 3	Day 4
Multiplicity of infection: 0.05			
Prague A	$5.6 \times 10^5$	$1.3 \times 10^6$	$3.2 \times 10^6$
Prague A + ascorbate	$0.35 \times 10^5$	$0.3 \times 10^6$	$1.2 \times 10^6$
% Inhibition	95	77	62
Multiplicity of infection: 5.0			
Prague A	$13.6 \times 10^6$	$24.0 \times 10^6$	$20.0 \times 10^6$
Prague A + ascorbate	$0.8 \times 10^6$	$1.3 \times 10^6$	$7.0 \times 10^6$
% Inhibition	94	95	62

PAT cells were infected in the absence of ascorbic acid. Medium was changed 4 hr after infection and ascorbic acid ( $50 \mu\text{g/ml}$ ) was added at that time. The result is the average of duplicate plates of three experiments.

\* Focus-forming units collected from 1 ml of medium per mg of cellular protein.

TABLE 3

Table 3. Ascorbic acid inhibition of infectious virus particles in PAT and CEF cultures

Culture	-Asc →	-Asc →	+Asc →	+Asc →
	-Asc	+Asc	-Asc	+Asc
<b>PAT</b>				
(FFU/mg protein)*	$7.6 \times 10^5$	$2.6 \times 10^5$	$4.9 \times 10^5$	0
% Inhibition†	—	64	35	>99
<b>CEF</b>				
(FFU/mg protein)	$3.1 \times 10^5$	$1.1 \times 10^5$	$0.7 \times 10^5$	$0.3 \times 10^5$
% Inhibition	—	65	78	91

Cells were grown with or without ascorbic acid (Asc) for 24 hr. One hour after infection with Prague C subgroup of RSV (multiplicity of infection = 1), half of each set was shifted with or without ascorbic acid. Medium was replaced 48 hr later and collected for focus assay after 2 hr. Results are the averages of duplicate plates.

\* FFU, focus-forming units.

† -Asc → -Asc titers were considered to be 100%.

TABLE 4

Table 4. Pretreatment of focus assay plates with ascorbic acid

Cultures	Exp. I		Exp. II	
	FFU/ml	Average focus size	FFU/ml	Average focus size
Control monolayer	$1.9 \times 10^7$	++++	$2.5 \times 10^6$	++++
+ ascorbate	$2.2 \times 10^7$	++	$4.0 \times 10^6$	++

Secondary cultures of CEF were made from primary cells that were grown with or without ascorbic acid for 4 days. Cells were seeded with or without ascorbic acid ( $50 \mu\text{g/ml}$ ) for 24 hr longer. The monolayers were infected in the absence of ascorbic acid with serial dilutions of the virus stock (Prague A grown in the absence of ascorbate). The agar overlay of the focus assay with ascorbate contained  $50 \mu\text{g}$  of ascorbic acid per ml. The largest foci (about 1–3 mm in diameter) were ++++; others were determined accordingly. FFU, focus-forming units.