Loss of GSH, Oxidative Stress, and Decrease of Intracellular pH as Sequential Steps in Viral Infection*

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Madin-Darby canine kidney cells infected with Sendai virus rapidly lose GSH without increase in the oxidized products. The reduced tripeptide was quantitatively recovered in the culture medium of the cells. Since the GSH loss in infected cells was not blocked by methionine, a known inhibitor of hepatocyte GSH transport, a nonspecific leakage through the plasma membrane is proposed. UV-irradiated Sendai virus gave the same results, confirming that the major loss of GSH was due to membrane perturbation upon virus fusion. Consequent to the loss of the tripeptide, an intracellular pH decrease occurred, which was due to a reversible impairment of the Na+/H+ antiporter, the main system responsible for maintaining unaltered pH in those cells. At the end of the infection period, a rise in both pH, value and GSH content was observed, with a complete recovery in the activity of the antiporter. However, a secondary set up of oxidative stress was observed after 24 h from infection, which is the time necessary for virus budding from cells. In this case, the GSH decrease was partly due to preferential incorporation of the cysteine residue in the viral proteins and partly engaged in mixed disulfides with intracellular proteins. In conclusion, under our conditions of viral infection, oxidative stress is imposed by GSH depletion, occurring in two steps and following direct virus challenge of the cell membrane without the intervention of reactive oxygen species. These results provide a rationale for the reported, and often contradictory, mutual effects of GSH and viral infection.

Oxidative stress in biological systems is caused by overload of oxidants, in particular reactive oxygen species, with respect to the antioxidant defense system developed by cells to counteract oxidation (1, 2). In fact, sustained oxidative insult tends to disrupt cell structures and functions, insofar as they are maintained and mediated by critical redox balance. Oxyradicals are suspected to be involved in several pathological processes such as inflammation, cancer, and neurodegenerative diseases (3), and, consequently, countering molecules are the object of intensive study. A major role in this respect has been assigned to the tripeptide glutathione, the most abundant non-protein thiol of the cell (4). It performs various functions ranging from cellular metabolism to transport as well as protection against free radicals and reactive oxygen species. The antioxidant function of the tripeptide is related to oxidation of the thiol group of its cysteine residue with formation of a disulfide (GSSG), which is, in turn, catalytically reduced back to the thiol form (GSH) by glutathione reductase.

In the past few years, in addition to detrimental effects, a metabolic requirement for oxygen radical-induced oxidative stress has been proposed. Evidence has accumulated suggesting that redox mechanisms play a fundamental role in cellular events such as binding of nuclear transcription factors to DNA, protein binding to mRNA, and hormone-receptor interactions. The most striking example is the redox regulation of transcription factors such as NF-κB and AP-1, which activate gene transcription in response to peroxide (5, 6).

It has also been reported that the nuclear factor κB is involved in the activation of HIV1 gene transcription (7). Therefore, it seems conceivable that oxidative stress might be a control factor in HIV infection. Furthermore, an altered antioxidant status was observed in plasma and other tissues of AIDS patients (8). However, the role played by oxidative conditions in HIV infection is still debated, on the basis of contradictory results. In fact, glutathione levels have been reported to decrease upon infection with HIV (9). The decrease seems to affect preferentially GSH, since relatively normal levels of GSSG were found in tissues of HIV-infected patients. On the contrary, another study reported an increase of GSSG, as mediator of oxidative stress, in CD4+ lymphocytes (10). Recently, it has also been suggested that GSH depletion is not directly related to infection of HIV virus in view of the lack of changes in the tripeptide content in circulating cells from AIDS patients (11).

Moreover, oxidative stress has been reported to occur in several different viral infections both in vivo (e.g. influenza (12, 13)) and in vitro (e.g. parainfluenza and herpes simplex type 1 (14, 15)).

In the search of a common redox event affecting virus replication, it has to be taken into account that the plasma membrane represents the earliest target of virus attack. It has been reported that oxidative stress is able to modify the activity of cell membrane ion transport systems, such as Ca2+ movements, Na,K-pump, and sodium, potassium, and chloride co-transport activity (16, 17). These ion transport systems are essential for proper cellular function and viability, and their activity is dependent on intracellular pH, which can be affected by GSH depletion.

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1 The abbreviations used are: HIV, human immunodeficiency virus; MDCK, Madin-Darby canine kidney; BCECF/AM, 2’,7’-bis(carboxyethyl)-5(6)-carboxyfluorescein, NaBH4, sodium borohydride; BSO, buthionine sulfoximine; DTT, dithiotreitol; DTNB, 5,5’-dithio-bis(2-nitrobenzoic acid); EIPA, 5-(N-ethyl-N-isopropylammonium) iodide; HAU, hemagglutinating units; PBS, phosphate-buffered saline; pH, intracellular pH; LDH, lactic dehydrogenase enzyme; AIDS, acquired immunodeficiency syndrome; HPLC, high pressure liquid chromatography.

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alterations may represent an early disturbance following oxidant exposure. Strictly connected to the previously mentioned ion transport mechanisms is the Na`/H` antiporter. This is a plasma membrane protein that exchanges sodium and hydrogen ions according to their concentration gradient, whose main function is the regulation of intracellular pH and cell volume (18). The Na`/H` antiporter is therefore strictly dependent on the sodium gradient, fueled by the Na-pump. Furthermore, the Na`/H` antiporter is able to modulate cell growth and proliferation, and its activation, with a consequent increase of intracellular pH, can represent a first response of the cell to hormones and growth factors (19). Recently, it has been reported that oxidative stress, associated with GSH decrease, produces changes in the Na`/H` antiporter activity, thus resulting in a significant decrease in the rate of recovery from an acid load (20).

In a previous study, we demonstrated that a loss in reduced glutathione occurs during the infection of epithelial cells with Sendai and herpes simplex type 1 (15, 21) viruses at very early stages of replication. In the present report we investigated the replication of Sendai virus in Madin-Darby canine kidney (MDCK) cells. Adsorption of virus to the cell appeared to be responsible for a major loss of GSH; GSH loss oxidatively affected the function of the Na`/H` antiporter, leading to lower intracellular pH. Since acidic conditions have been demonstrated to favor early stages of viral replication (22, 23), a more general relationship between virus replication and oxidative stress may be found in the events following GSH depletion.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**MDCK cells were purchased from the American Type Culture Collection and grown in RPMI 1640 supplemented with heat-inactivated 5% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Vitality was estimated by trypan blue exclusion, and cell density was determined using a Neubauer chamber. RPMI 1640 minimal growth medium, fetal calf serum, glutamine, penicillin, and streptomycin were purchased from Flow Laboratories. 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF/AM) was purchased from Molecular Probes, Inc. Nigericin, 2,4-dinitrobenzene, sodium borohydride (NaBH₄), buthionine sulfoximine (BSO), dithiothreitol (DTT), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and dimethyl sulfoxide (Me₂SO) were from Sigma. 5-N-ethyl-N-isopropylamiloride (EIPA) was from Research Biochemicals, Inc. L-methionine, GSH, GSSG, pyruvate, and Na⁺/H⁺ were from Boehringer Mannheim. [³⁵S]cysteine (37 Bq/mk) was from Amersham Interna-

tional. Goat anti-parainfluenza antibodies were purchased from Biodesign International. Chamber slides were from Lab-Tek, Nunc, Inc. All other chemicals were obtained from Merck.

**Virus Infection and Titration—**Sendai virus is a nonsegmented single-stranded RNA virus of negative polarity. Stocks of the virus were obtained by allantoic inoculation of 10-day-old embryonated eggs with 0.2 ml of a 10⁻¹ dilution of infected allantoic fluid. Confluent cultures of cells were infected with 10 hemagglutinating units (HAU)/2 × 1₀⁵ cells. After incubation for 1 h at 37°C (adsorption period), unadsorbed virus was removed, and the monolayers were washed three times with PBS. Virus production was determined by measuring the number of hemagglutinin units present in the medium of infected monolayers of MDCK cells at different times postinfection.

In some experiments, cells were prewashed with ice-cold PBS and either mock-infected or infected with Sendai at 4°C in order to avoid the fusion of the virus with the cell membranes. In other experiments, we infected the cells with UV-inactivated virus, which is unable to replicate as the consequence of UV-induced RNA damage. Virus suspensions were exposed to UV light (40 W, 254 nm, 8 cm distance) for 10 min. After neutralization with a solution of 3% sodium bicarbonate, virus suspensions were exposed to UV light (40 W, 254 nm, 8 cm distance) for 10 min. After neutralization with a solution of 3% sodium bicarbonate, virus suspensions were washed twice with PBS and sonicated in a water bath sonicator to break up virus aggregates. Titration of purified virus was performed by measuring the number of hemagglutinin units present in the medium of infected monolayers of MDCK cells at different times postinfection.

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### Intracellular pH Measurements

**Intracellular pH**

- **Determination of Glutathione and Mixed Disulfides—**Intracellular glutathione was assayed upon formation of S-carboxymethyl derivatives of free thiols with iodoacetic acid following conversion of free amino groups to 2,4-dinitrophenyl derivatives by reaction with 1-fluoro-2,4-dinitrobenzene as described by Reed et al. (25). Monolayers of MDCK cells were carefully washed with a large volume of PBS. The cells were detached by gentle scraping and centrifuged at 700 × g for 10 min. The supernatant was discarded, and the cell pellet was resus- pended in 200 μl of buffer. Cell lysis was obtained by repeated cycles of freezing and thawing under liquid nitrogen. Protein was then precipitated by adding metaphosphoric acid (5% final concentration). After centrifugation at 22,300 × g for 30 min, the low molecular thiols in the supernatant were derivatized and measured by HPLC using a µBondapak NH₂ column (Waters). GSH and GSSG were used as external standards. Mixed disulfides were determined by HPLC after reduction of cell lysates with NaBH₄ for 30 min at 4°C (20). GSH and GSSG in the culture medium were determined after centrifugation of the collected media at 900 × g for 10 min and subsequent acidification.

A aliquot of cell lysates was used for total protein determination by the method of Lowry et al. (27).

**Lactic dehydrogenase enzyme activity (LDH)** was determined as described (26) both in the culture medium and intracellularly. Control or infected cells were gently scraped in the culture medium. An aliquot of the cell suspension was used for total enzyme assay after cell lysis. Another aliquot was centrifuged at 700 × g for 10 min. The supernatant was then used for extracellular enzyme assay. The extracellular enzyme was expressed as the percentage of the total activity.

**Cell Treatments—**Confluent monolayers were treated with 0.5 mM DTT or 1 mM DTNB for 24 h after virus infection. BSO (1 mM) was added to cells 18 h before virus infection, and/or for 24 h after. At the end of the treatments, cells were washed twice with PBS for virus titration, and cells were washed, scraped, and pelletted by centrifugation at 700 × g for 10 min and treated for GSH determination. All substances were used at nontoxic concentrations, as evaluated by the trypan blue exclusion test.

**In some experiments monolayers were treated with 1 μM methionine 1 h before virus infection and for 24 h after.**
conditions cells were considered bicarbonate-free. Cells were then incubated in Na\textsuperscript{1} buffer with the fluorescent dye (1 mg/ml in Me\textsubscript{2}SO) at a final concentration of 1 \mu g of BCECF/10\textsuperscript{6} cells, for 30 min at 37 °C in the dark. Then the medium containing the dye was eliminated, and the cells were washed twice with the same buffer.

The calibration curve was carried out as previously reported (32) using the nigericin (10 mM in ethanol) method in a medium containing high potassium with same composition as Na\textsuperscript{1} medium but equimolar KCl substituted for NaCl. The calibration curve was linear in the range of pH 6.5–7.5 (not shown).

Fluorescence was also measured under continuous magnetic stirring and with controlled temperature (37 °C) in a Perkin-Elmer luminescence spectrometer LS-5 equipped with a chart recorder model R 100A, with excitation and emission wavelengths of 500 and 530 nm, using 5 and 10 nm slits, respectively, for the two light pathways. Fluorescence was also routinely measured at 450-nm excitation (at this wavelength the fluorescence is proportional to intracellular dye concentration but is relatively pH-insensitive), and the value did not change more than 5% during the experimental period.

\textbf{Determination of Intrinsic }\beta_i—\beta_t = \beta_{\text{CO}_2} + \beta_i, \text{ where } \beta_i \text{ is the total intracellular buffering capacity, but in the nominally } \text{HCO}_3^- \text{ -free solutions used in this study, } \beta_{\text{CO}_2} \text{ was assumed to be negligible, and } \beta_i \text{ was therefore taken to be equal to } \beta_i. \beta_i \text{ was determined using the } \text{NH}_4^+ \text{ pulse technique as described previously (33) according to the formula,}

\[
[N\text{H}_4^+] = [N\text{H}_4^+]_0 \cdot 10^{8.92-p\text{H}} \tag{1}
\]

taking into account that \text{NH}_4 equilibrates across the cell membrane \textit{i.e.} \text{[NH}_4^+]_o = [N\text{H}_4^+]_i \text{ and that the pK}_a \text{ of } \text{NH}_4^+ (8.92) \text{ is the same intra- and extracellularly. } [N\text{H}_4\text{Cl}] \text{ in the absence of } N\text{H}_4\text{Cl was taken to be 0.}

Statistical analyses were performed using Student’s \textit{t} test for unpaired data, and \textit{p} values <0.05 were considered significant. Data are presented as mean ± S.D.

\textbf{RESULTS}

\textbf{Effect of Virus Adsorption on the Glutathione Status—MDCK} cells infected with Sendai virus show a time-dependent decrease in the intracellular GSH content (Fig. 1). GSH decrease began after a few minutes of infection and reached its maximum value in 20 min during virus adsorption (\textit{p} < 0.001). At the end of the virus infection (1 h) GSH content tended to increase; however, the values were always significantly lower than those observed in control cells (Fig. 1). A further decrease was observed in the following 24 h (Fig. 1, inset). In particular, the loss of GSH 3 h after infection was highly significant (\textit{p} < 0.001) with respect to 1 h. At the end of 24 h, virus particles were detectable in the supernatant of infected cells, indicating that the virus had completed its replication. The same results were obtained by infecting the cells with purified Sendai virus. On the contrary, changes in the GSH content were not observed when treating the cells with allantoic fluid from uninfected cells (mock infection) at the same virus dilution (data not shown).

Since GSH decrease was a two-step process, we performed the experiments at 20 min, in the early phase of virus infection, and at 24 h, at the late stage of virus replication. In particular, 20 min referred to monolayers infected with Sendai virus only for this length of time; 24 h referred to cells infected for 1 h and reinfected with fresh medium for an additional 24 h.

GSH depletion is commonly observed when cells are oxidatively stressed. It can usually be detected by measurement of transient increases in the intracellular content of either GSSG or protein glutathione mixed disulfides. To assess whether a typical oxidative burst was taking place under our experimental conditions, we measured the intracellular GSSG content and the GSH equivalents bound to proteins.

However, despite a massive and rapid GSH decrease, we were unable to detect any change in the concentrations of the two oxidized GSH derivatives after 20 min of incubation of MDCK cells with Sendai virus. The GSSG value in infected

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Effect of Sendai virus infection on intracellular GSH content of MDCK cells. Confluent monolayers were infected with Sendai virus (10 HAU/2 × 10\textsuperscript{5} cells). Cells were collected at various times, and intracellular GSH content was measured as described under “Experimental Procedures.” Data points show mean ± S.D. (\textit{n} > 10). All data points are significantly different from uninfected cells (\textit{p} < 0.001). *, significantly different from 1 h; \textit{p} < 0.001.}
\end{figure}
treated with 1 mM buthionine sulfoximine, a specific inhibitor and subsequent resynthesis. Under our conditions the fall in efflux out of the cell or a reduced rate of uptake of precursors between the rates of synthesis and loss of the tripeptide via redox form within the cell.

20 min. This indicates that either the rate of GSH extrusion is of glutathione synthesis, show a 50% decrease of GSH only after 18 h of treatment.

On the other hand, large amounts of glutathione were detected in the media of infected cells (Table I), predominantly as GSH (90%). Since the medium derived from infected cells was not able to reduce externally added GSSG (data not shown), it was concluded that during virus infection, glutathione is released by the cell in its reduced form. Furthermore, changes in the GSH content of the culture medium were not observed upon incubation with 5 mM DTT for 1 h, before acidification.

A steady state level of GSH in the cell results from a balance between the rates of synthesis and loss of the tripeptide via oxidation or excretion. A declining in GSH levels in the absence of oxidation can potentially result either from an increased efflux out of the cell or a reduced rate of uptake of precursors and subsequent resynthesis. Under our conditions the fall in GSH was too rapid to be accounted for by an inhibition of precursor uptake or biosynthesis. Moreover, MDCK cells treated with 1 mM buthionine sulfoximine, a specific inhibitor of glutathione synthesis, show a 50% decrease of GSH only after 18 h of treatment.

In order to address the question whether GSH efflux from cells occurs by leakage or by carrier-mediated transport, we measured the activity of lactic dehydrogenase enzyme in the media of infected cells as an indicator of cell membrane perturbation. The data reported in Fig. 2A clearly demonstrate that outside enzyme activity can be detected at 20 min. However, the values represent only a small amount of the total enzyme present intracellularly, 2–3%. As assessed by trypan blue exclusion, cells were 98–100% viable, the same level observed in uninfected cells.

An increase in the activity of this enzyme was also observed in the following 24 h of infection in the media of both control and infected cells. In this case the enzyme activity assayed in infected media was not significantly different from control media (Fig. 2B).

Several GSH-specific transporters were identified on the cellular and mitochondrial membranes of different cellular systems. These are specifically inhibited by L-methionine or cystathionine. MDCK cells were treated with 1 mM methionine for 1 h before virus infection and for 24 h after infection. The rate of GSH efflux was decreased in uninfected cells; in fact, a 30 and 70% inhibition of the efflux rate was obtained at 80 min (1 h before and 20 min during infection) and 24 h, respectively (Fig. 3), with a slight increase (10–15%) in the intracellular GSH content at 80 min. However, the inhibitory effect was not observed in the infected cells at either 20 min or 24 h (compare Tables I and II with Fig. 3), in line with the result of an unaltered virus production obtained at 24 h as reported in Fig. 3, inset.

The results obtained point to a carrier-independent GSH extrusion. To further investigate this phenomenon, two different approaches were adopted. One approach was to infect MDCK cells at 4°C, a condition under which the virus is adsorbed to the cells but is not able to fuse with them. The other approach was to infect cells with Sendai virus unable to replicate because inactivated by UV radiation. GSH loss at 20 min was not observed when cells were infected with Sendai virus at 4°C (data not shown). On the contrary, the same extent of loss, in terms of GSH content and LDH activity, was observed when cells were infected with Sendai virus previously

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**Table I**

|               | GSH        | GSSG        | Mixed disulfides | GSH        | GSSG        |
|---------------|------------|-------------|------------------|------------|-------------|
| Intracellular |            |             |                  |            |             |
| Control (n > 10) | 31.09 ± 2.74 | 0.38 ± 0.14 | ND               | 0.92 ± 0.06 | ND          |
| Infected (n > 10) | 16.42 ± 1.94b | 0.30 ± 0.13 | ND               | 6.40 ± 1.22b | 1.15 ± 0.18 |

* ND, not detectable.

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**Table II**

|               | GSH        | GSSG        | Mixed disulfides | GSH        | GSSG        |
|---------------|------------|-------------|------------------|------------|-------------|
| Intracellular |            |             |                  |            |             |
| Control (n > 10) | 26.33 ± 3.73 | 0.43 ± 0.15 | ND               | 12.54 ± 4.05 | 1.85 ± 0.21 |
| Infected (n > 10) | 9.84 ± 2.01b | 0.24 ± 0.10b | 0.96 ± 0.15      | 18.86 ± 5.02c | 2.28 ± 0.25 |

* ND, not detectable.

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A steadystate level of GSH in the cell results from a balance between the rates of synthesis and loss of the tripeptide via oxidation or excretion. A declining in GSH levels in the absence of oxidation can potentially result either from an increased efflux out of the cell or a reduced rate of uptake of precursors and subsequent resynthesis. Under our conditions the fall in GSH was too rapid to be accounted for by an inhibition of precursor uptake or biosynthesis. Moreover, MDCK cells treated with 1 mM buthionine sulfoximine, a specific inhibitor of glutathione synthesis, show a 50% decrease of GSH only after 18 h of treatment.

In order to address the question whether GSH efflux from cells occurs by leakage or by carrier-mediated transport, we measured the activity of lactic dehydrogenase enzyme in the media of infected cells as an indicator of cell membrane perturbation. The data reported in Fig. 2A clearly demonstrate that outside enzyme activity can be detected at 20 min. However, the values represent only a small amount of the total enzyme present intracellularly, 2–3%. As assessed by trypan blue exclusion, cells were 98–100% viable, the same level observed in uninfected cells.

An increase in the activity of this enzyme was also observed in the following 24 h of infection in the media of both control and infected cells. In this case the enzyme activity assayed in infected media was not significantly different from control media (Fig. 2B).

Several GSH-specific transporters were identified on the cellular and mitochondrial membranes of different cellular systems. These are specifically inhibited by L-methionine or cystathionine. MDCK cells were treated with 1 mM methionine for 1 h before virus infection and for 24 h after infection. The rate of GSH efflux was decreased in uninfected cells; in fact, a 30 and 70% inhibition of the efflux rate was obtained at 80 min (1 h before and 20 min during infection) and 24 h, respectively (Fig. 3), with a slight increase (10–15%) in the intracellular GSH content at 80 min. However, the inhibitory effect was not observed in the infected cells at either 20 min or 24 h (compare Tables I and II with Fig. 3), in line with the result of an unaltered virus production obtained at 24 h as reported in Fig. 3, inset.

The results obtained point to a carrier-independent GSH extrusion. To further investigate this phenomenon, two different approaches were adopted. One approach was to infect MDCK cells at 4°C, a condition under which the virus is adsorbed to the cells but is not able to fuse with them. The other approach was to infect cells with Sendai virus unable to replicate because inactivated by UV radiation. GSH loss at 20 min was not observed when cells were infected with Sendai virus at 4°C (data not shown). On the contrary, the same extent of loss, in terms of GSH content and LDH activity, was observed when cells were infected with Sendai virus previously

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The glutathione status of MDCK cells infected with Sendai virus

Glutathione status of MDCK cells infected with Sendai virus

Infection was carried out for 20 minutes at 37 °C. Medium was then collected and treated as described under “Experimental Procedures” for the GSH assay. Monolayers were then washed and treated for GSH assay as described under “Experimental Procedures.”

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inactivated (Fig. 4). Moreover, the GSH value present in the medium at 24 h was the same as that of control cells, indicating that, as expected, the UV-irradiated virus was not able to replicate. This was confirmed by the absence of hemagglutinating activity in the media (data not shown).

Effect of the Modulation of Glutathione Status on Virus Replication—To investigate the correlation between the change in intracellular redox state, consequent to GSH loss, and virus replication, we treated the cell with different agents, able to modulate the intracellular GSH status, and subsequently quantified the changes in virus yield. Data shown in Fig. 5 indicate that the treatment of cells with 1 mM BSO for 18 h before infection and 24 h after caused an 80% decrease in GSH. This decrease was reflected by a double yield in virus production by cells. The opposite effect has been already shown in a previous work (21); treatment of cells with exogenous GSH, which is able to maintain unaltered the intracellular GSH content, inhibited virus replication by 90%. Moreover, incubation of the monolayers with 0.5 mM DTT, a reducing agent, during virus replication lowered significantly the virus titer and increased the GSH content (Fig. 5). On the contrary, 1 mM DTNB, a sulfhydryl reagent, increased viral production without significant change in the GSH content of the cells (Fig. 5).

Effect of Viral Infection and Glutathione Depletion on the Steady-state pH of MDCK Cells—Chemical depletion of intracellular GSH produced a decrease in cellular pH in Ehrlich tumor cells (34). Moreover, lower intracellular pH values are preferred by several viruses for efficient replication (22, 23). We therefore evaluated whether changes in the cells’ pH value were operative during Sendai virus infection.

In the standard HEPES-buffered bicarbonate-free solution, the mean steady-state pH of MDCK cells was 7.25 ± 0.07 (n = 15). A short term infection (20 min) of MDCK cells gave rise to a decrease of intracellular pH of about 0.20 pH units, whereas an infection ranging from 1 h up to 24 h did not give any significant change in basal intracellular pH evaluated as reported by Thomas et al. (32) (Table III). GSH depletion achieved by 18-h treatment with 1 mM BSO was similar to that obtained by 20-min infection and at the same time gave rise to a significant decrease of intracellular pH, in the same range as a 20-min viral infection (Table III).

Recovery of pH from an Acid Load: Effect of Viral Infection—In order to assess whether the decrease of intracellular pH observed either with virus treatment or with chemical GSH depletion was due to an effect on the Na⁺/H⁺ antipporter, we studied the ion transport activity under conditions of maximum activation, after an acid load.

The exposure of MDCK cells to 20 mM NH₄Cl-containing HEPES-buffered solution increased pH rapidly to approximately 0.30 pH units over the basal value (Figs. 6A and 7A), via rapid diffusion of NH₃. During the exposure to NH₄Cl (about 4 min), pH tended to decrease toward the base line due to slow inward diffusion of NH₄⁺. Abrupt removal of NH₄Cl from the medium rapidly decreased pH by approximately 1.00 pH unit, with respect to the base line, due to NH₄ leaving the cell. The extent of this acidification was the same in all monolayers during the NH₄Cl washout.

The readdition of Na⁺ buffer caused a recovery of pH, within about 10 min, in agreement with previously published data (35) and with a time course normally fitted by a single exponential.
GSH Loss and Viral Replication

Fig. 4. GSH content of MDCK cells infected with UV-irradiated Sendai virus. Monolayers were infected with Sendai virus previously inactivated by UV irradiation as described under “Experimental Procedures.” GSH content and LDH activity were determined as under Figs. 1 and 2. C, control cells; UV, cells infected with UV-irradiated Sendai virus. n = 10; *, p < 0.001.

Fig. 5. Effect of modulation of GSH status on Sendai virus replication. Monolayers were treated with 1 mM BSO for 18 h before infection and for 24 h after. 0.5 mM DTT or 1 mM DTNB were added to the cells only for 24 h after infection. Cells and culture medium were treated for GSH and HAU determinations as described under “Experimental Procedures.” One experiment is reported of six performed with similar results.

Therefore, a plot of ΔpH/Δt (dpH/dt) versus pHi is a straight line, intersecting the pHi axis at the final steady-state pHi (Figs. 6B and 7B).

In MDCK cells infected for 20 min, the rate of recovery was decreased, and the final pHi did not reach the initial value (Fig. 6A), whereas a 24-h viral infection did not give rise to any significant change in the recovery of intracellular pH (not shown), but only a trend to a decrease in the set point was observed (Table III and Fig. 6B). The same panel also shows the lack of recovery both in control and in 20-min infected cells due to EIPA (20 μM), a derivative of amiloride, a specific inhibitor of the Na+/H+ antiporter. The sensitivity to the amiloride derivative confirms that we are dealing with the basolateral Na+/H+ antiporter having a housekeeping function (36). The regression lines, in the range 6.2–7.4, computed from data of experiments similar to those reported in Fig. 6A and 7A are reported in Fig. 6B as mean ΔpH (× 10–4 pH units)/Δt (s) (dpH/dt) versus pHi during recovery from NH4·NH4 pulse in MDCK cells. The regression lines for control and infected cells were parallel, but the set point of pHi was 7.29, 7.04, and 7.24 for control, 20-min infected, and 24-h infected cells, respectively (Fig. 6B). The same type of result was achieved when cells were depleted of GSH by BSO treatment (Fig. 7). In the case of BSO-treated cells, the slope of the regression line was also affected, in addition to the set point, with respect to control (Fig. 7B). The set point of pHi was 7.28 and 7.01 for control and BSO-treated cells, respectively, in quite good agreement with previously published literature for the same cells (35), whereas an acute infection of MDCK cells resulted in an impairment of the Na+/H+ antiporter function as shown by the lack of recovery from an acid pulse.

Cysteine Uptake—The preceding results demonstrate that GSH decreases during Sendai virus replication but with a less significant increase in the culture medium than that observed in the 20-min infection. Therefore, GSH metabolism in MDCK cells during virus replication was further investigated by labeling the intracellular GSH pool with [35S]cysteine. During the 24-h labeling period, control and infected cells took up the same amount of [35S], with significant changes in the trichloroacetic acid-soluble material; indeed, a decrease was observed in infected cells (~34%) (Table IV), in line with the intracellular GSH determinations. Furthermore, a significant amount of [35S] was found to be associated with viral proteins, as expected from the knowledge that Sendai envelope proteins contain a large number of cysteine residues. Interestingly, the loss of label in the acid-soluble material of infected cells was almost quantitatively recovered in the immunoprecipitated viral proteins (Table IV).

DISCUSSION

The results reported above demonstrate that GSH depletion is a direct consequence of viral infection.

It is usually assumed that GSH depletion reflects an intracellular oxidation; however, the results presented here suggest an alternative mechanism. GSH was lost from cells undergoing viral infection in a two-step process, it was mainly leaked out the plasma membrane rather than depleted by an oxidative process, as confirmed by unaltered intracellular GSSG content. A large amount of the tripeptide in its reduced form was, in fact, detected in the cell culture medium a few minutes after infection. The very rapid GSH loss from cells implied that the consequent intracellular decrease was not due to an inhibition in the uptake of GSH precursors and subsequent impairment of the synthesis. Moreover, chemical inhibition of the GSH synthesis by BSO led to very slow GSH decrease (50% reduction was reached by at least 18 h of treatment).

GSH decrease at the very early stages of virus infection was also observed during the infection of VERO cells with clinically isolated herpes simplex type 1 virus (15), indicating a more general relationship between oxidative stress and viral replication.

Sinusoidal efflux of GSH in the liver is markedly inhibited by low concentrations of methionine, and such inhibition is taken as evidence for the presence of a specific GSH carrier (37). We found that uninfected MDCK cells also extruded GSH, as shown by the increased thiol concentration in the medium of 24 h cultured cells, by a methionine-sensitive mechanism. At
variance with this, no inhibition by methionine was observed in infected cells, ruling out an active extrusion process. Further, inhibiting the fusion process by infecting the cells at 4°C did not result in changes in GSH content. On the contrary, inhibiting viral replication by a preinactivation of Sendai virus by UV irradiation gave rise to changes, at 20 min, comparable with those observed in cells infected by the active virus, confirming that the major loss of GSH was associated with the fusion process.

The GSH loss observed at 20 min imposes an oxidative stress that can impair other cellular functions, in particular those regulated by the redox mechanism. We found that GSH leakage was associated with a decrease in intracellular pH, consistent with a previous study that demonstrated lower intracellular pH value in Ehrlich-ascites tumor cells chemically depleted of GSH (34). Moreover, under our conditions, an 18-h treatment of monolayers with BSO reduced the intracellular GSH to values similar to those mediated by a 20-min virus infection, leading to a concomitant decrease in the pH.

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**TABLE III**

**Effects of viral infection and BSO treatment on the resting intracellular pH in MDCK cells**

Results are means ± S.D. of experiments whose number is reported in parentheses. BSO (1 mM) treatment was carried out for 18 h.

|        | Control 20 min | 1 h | 3 h | 24 h | BSO |
|--------|---------------|-----|-----|------|-----|
| pH     | 7.25 ± 0.07 (15) | 7.03 ± 0.10 (6) | 7.20 ± 0.10 (4) | 7.24 ± 0.09 (4) | 7.18 ± 0.11 (9) | 7.10 ± 0.08 (3) |

*a p < 0.01 with respect to control.*

**FIG. 6.** Effect of Sendai virus infection on the pH_i recovery from an acid load with NH_4^+ pulse in MDCK cells. A, after a 2-min equilibration in sodium buffer, at time 0 cells were exposed to 20 mM NH_4Cl for ~4 min. During this time the intracellular pH increased (due to entry of NH_3 into the cell) and then decreased toward the baseline due to the slower influx of NH_4^+. Then the NH_4Cl was removed, and the sodium buffer was reintroduced. B, regression lines calculated from the recovery rates of experiments reported as representative in A. The lines are means of n = 15 for control (○), n = 6 for 20-min infected (●), and n = 9 for 24-h infected (■). The graphs are computer-generated regression lines whose parameters are as follows: control, a = 285.03 ± 17.34 × 10^−4 pH units/s, b = −0.025 s^−1, and a computed x intercept of 7.29; 20-min infected, a = 249.32 ± 23.49 × 10^−4 pH units/s, b = −0.028 s^−1, and a computed x intercept of 7.04; 24-h infected, a = 262.80 ± 16.56 × 10^−4 pH units/s, b = −0.027, and a computed x intercept of 7.25.
A wide variety of external signals enhance the affinity of the antiporter for H\(_1\) at neutral pH, leading to alkalinization of the cells. MDCK cells were found to regulate their pH\(_i\) primarily via the Na\(^+\)/H\(^+\) antiporter, as demonstrated by the ability of EIPA to inhibit recovery from an acid load (Fig. 6A). Such recovery was not observed in infected cells at 20 min, suggesting that a modification of the Na\(^+\)/H\(^+\) antiporter was responsible for the acidification occurring at the early stage of infection. Interestingly, cells treated with BSO showed the same impairment in the function of the antiporter. In this context it has to be mentioned that oxidative stress decreases the Na\(^+\)/H\(^+\) antiporter activity in bovine pulmonary artery endothelial cells (20).

It has been demonstrated that acidic conditions favor many viral infections by accelerating the fusion process, thus enhancing viral replication (22, 23). Sendai virus (hemagglutinating virus of Japan) belongs to the Paramyxovirus family and is believed to infect its host cells directly by fusion of its envelope with the cell plasma membrane (39). The rate of fusion of Sendai with several cellular systems was found to be optimal at pH 7.0 (40). This was in good agreement with the drop of pH\(_i\) observed at 20 min of infection. Furthermore, by decreasing intracellular GSH and pH\(_i\) upon BSO treatment we obtained 2 times higher virus production in 24 h. These results confirm that a lower pH\(_i\) value in comparison with that observed in control cells favors Sendai virus replication.

Despite the large loss of GSH and the consequent severe impairment in the Na\(^+\)/H\(^+\) antiporter, cell death was not ob-

**TABLE IV**

| Total uptake | Trichloroacetic acid-soluble material | Viral proteins |
|--------------|-------------------------------------|---------------|
| cpm × 10\(^3\) | cpm × 10\(^3\) | cpm × 10\(^3\) |
| Control      | 32.3 ± 4.3 | 7.1 ± 0.7 | 0.3 ± 0.4 |
| Infected     | 27.4 ± 2.1 | 4.6 ± 1.0\(^a\) | 1.7 ± 0.1 |

\(^a\) p = 0.01.

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Despite the large loss of GSH and the consequent severe impairment in the Na\(^+\)/H\(^+\) antiporter, cell death was not ob-
served in infected cells, even if there is convincing evidence
that T lymphocytes infected with HIV exhibit an enhanced
susceptibility to undergo apoptosis (programmed cell death)
(41). Under our conditions MDCK cells are still viable after
48–72 h of infection. A recovery from the initial oxidative
conditions was observed at 1 h of infection as evidenced by the
rise in both intracellular pH and GSH content. In fact, 1 h
 corresponds to the end of the viral adsorption period, thus
confirming the strict association of early infection steps with
 GSH loss and pH drop.

A completely different scenario is operative during virus
replication. In this second phase of the virus growth program,
prooxidative conditions are still in action as shown by a further
GSH decrease. However, in this case GSH loss was not associ-