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## Effect of Sulfhydryl Reagents on the Infectivity of Vesicular Stomatitis Virus

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The infectivity of vesicular stomatitis virus (VSV) was moderately affected by iodoacetic acid and drastically affected by *N*-ethylmaleimide; the antiviral effect of these sulfhydryl reagents was enhanced somewhat by the reducing agent, 2-mercaptoethanol. Reducing and/or alkylating reagents did not affect VSV hemagglutination and the impermeable sulfhydryl reagent, dextran-maleimide, did not significantly influence VSV infectivity. These data indicate that glycoprotein spikes are not the major sites for the antiviral activity of sulfhydryl reagents. [<sup>14</sup>C]Iodoacetic acid was able to penetrate the virion membrane to bind covalently to the free sulfhydryl groups of all five virion proteins, particularly the reduced disulfides of the L protein. The RNA polymerase activity of intact VSV was inhibited by iodoacetic acid and to a greater extent by *N*-ethylmaleimide, which probably accounts for the loss of viral infectivity caused by the permeable sulfhydryl reagents.

Significant biological functions have been attributed to the sulfhydryl groups and disulfide bonds of viral proteins. Early studies were directed to the effect of sulfhydryl blocking reagents on the capacity of viruses to initiate infection (Philipson and Choppin, 1960, 1962; Choppin and Philipson, 1961). It was found that a number of sulfhydryl binding reagents reduced the infectivity of various picornaviruses. A sulfhydryl reagent was also found to inactivate hemagglutination by a wide variety of enveloped viruses (Philipson and Choppin, 1960) as well as the infectivity of influenza virus although adsorption was not affected (Choppin and Philipson, 1961). Sulfhydryl reagents also lowered the infectivity of paramyxoviruses (Scheid and Choppin, 1974, 1977). More recent studies with measles virus (Hardwick and Bussell, 1978) have indicated the importance of disulfide-linked polypeptides for the biological activity of the surface glycoproteins.

We decided to investigate the biological

functions of the sulfhydryl groups and disulfide bridges of the well-characterized vesicular stomatitis virus (VSV). Several, if not all, of the five VSV proteins appear to have free sulfhydryl groups which can be oxidized to crosslink proteins by forming disulfide bonds (Dubovi and Wagner, 1977). We first focused our attention on the single surface glycoprotein of VSV because of its obvious role in the initiation of infection (Bishop *et al.*, 1977) and as the major antigenic determinant (Kelley *et al.*, 1972). Moreover, Kelley and Emerson (unpublished data) have uncovered evidence that three disulfide bridges contribute to the secondary structure of the VSV glycoprotein, thus making it a likely candidate for the effect of reducing and alkylating reagents. We soon discovered that SH groups of other VSV proteins appear to make more significant contributions to the biological activity of VSV.

### MATERIALS AND METHODS

The Indiana serotype (San Juan strain) of cloned VSV was produced by infecting BHK-21 cells at multiplicities <0.01; infectivity was titrated by plaque assay on

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TABLE 1

COMPARATIVE INFECTIVITY OF VS VIRUS EXPOSED TO THREE SULFHYDRYL BLOCKING REAGENTS IN THE ABSENCE OR PRESENCE OF 2-MERCAPTOETHANOL<sup>a</sup>

SH reagent concentration (mM)	Infectivity (PFU/ml)					
	IAA		NEM		Dextran-NEM	
	Alone	+2-ME	Alone	+2-ME	Alone	+2-ME
0	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$
0.01			$2 \times 10^8$	$1 \times 10^8$		
0.05			$1 \times 10^6$	$8 \times 10^5$		
0.1	$2 \times 10^9$	$2 \times 10^9$	$5 \times 10^5$	$2 \times 10^5$		
1.0	$2 \times 10^9$	$5 \times 10^8$	$3 \times 10^4$	$1 \times 10^4$	$1 \times 10^9$	$8 \times 10^8$
10.0	$1 \times 10^9$	$3 \times 10^7$	$<1 \times 10^2$	$<1 \times 10^2$	$5 \times 10^8$	$5 \times 10^8$
20.0	$7 \times 10^8$	$1 \times 10^7$				
50.0	$5 \times 10^8$	$5 \times 10^6$				
100.0	$3 \times 10^6$	$3 \times 10^5$				

<sup>a</sup> Purified VS virus preincubated for 30 min at room temperature alone or with 2-mercaptoethanol (2-ME equimolar to SH reagent) was incubated for 30 min at room temperature with varying concentrations of iodoacetic acid (IAA), *N*-ethylmaleimide (NEM), or dextran-maleimide (MW  $\approx$  10,000). Each treated or untreated control virus preparation was serially diluted 10-fold in PBS and assayed for infectivity by plating 0.2 ml of each dilution on duplicate monolayer cultures of L cells. Infectivity of VS virus was not affected (or slightly enhanced) by exposure to 2-ME alone (up to 100 mM) or to dextran alone (up to 10 mM).

monolayers of L cells (Wagner *et al.*, 1963). Virus purified by differential and rate zonal centrifugation (Dubovi and Wagner, 1977) was exposed for 30 min at 25° with either iodoacetic acid (IAA), *N*-ethylmaleimide (NEM) (Pierce Company, Rockford, Ill.), or dextran-maleimide which was kindly supplied by Agnete Faegri of this department, who synthesized the compound by the method of Abbott and Schacter (1976). The infectivity of reduced and/or alkylated VSV was determined by plating on L cells. Reduced and/or alkylated VSV repurified by centrifugation through a 50% glycerol cushion was assayed for polymerase activity after disruption in 2% Triton X-100 in buffered high salt solution, as previously described (Hunt and Wagner, 1975). The presence of free sulfhydryl groups or reducible disulfide bonds on VSV protein was determined by electrophoresis on 2% SDS-7.5% polyacrylamide gels (Dubovi and Wagner, 1977) after reacting reduced or unreduced virus with [<sup>14</sup>C]-iodoacetic acid (200 mCi/mmol) (New England Nuclear, Boston, Mass.); before electrophoresis the virus was dialyzed

overnight to remove free [<sup>14</sup>C]iodoacetic acid and was boiled for 2 min in 2% SDS. All reactions of VSV with sulfhydryl reagents were carried out in Tris-HCl buffer, pH 7.2.

## RESULTS

To determine whether free sulfhydryl groups and/or disulfide bonds influence infectivity, intact VS virions were exposed to IAA alone or after incubation of virions with 2-ME. The infectivity of VS virions was not reduced by exposure to 2-ME alone, even at a concentration of 100 mM; in fact, 2-ME slightly enhanced the plating efficiency of VSV (data not shown). The effect of IAA at various concentrations was determined by incubation at room temperature for 30 min with VSV without or after 30-min incubation with 2-ME at equimolar concentration.

Table 1 shows that the infectivity of unreduced VS virus was only slightly affected by IAA even at a concentration of 50 mM; only at a concentration of 100 mM did IAA inhibit infectivity markedly. Prior reduc-

tion of disulfide bonds by 2-ME resulted in greater susceptibility of VSV to inactivation by IAA. The data depicted in Table 1 indicate that increased acetylation of previously reduced disulfide bonds results in gradual decline in infectivity of VSV.

A possible explanation for loss in infectivity after reduction and/or alkylation of VSV might be attributed to alteration in the cell-surface receptor-binding function of the G protein (Bishop *et al.*, 1975). One way to measure the role of the G protein in adsorption to cell surfaces is by hemagglutination of goose erythrocytes (Schloemer and Wagner, 1975). To this end concentrated VSV, previously treated with PBS or 10 mM 2-ME, was exposed for 30 min at 25° to 10 mM IAA or 1 mM NEM. Each reduced and/or alkylated preparation of virus was then tested for its ability to agglutinate goose erythrocytes (Schloemer and Wagner, 1975). In all cases the virus completely retained its hemagglutinating titer of 1:32. These results indicate that the loss of VSV infectivity caused by 2-ME and IAA or by NEM is not attributable to significant alteration in the receptor-binding function of the G protein.

In much earlier studies Philipson and Choppin (1960) had shown that different sulfhydryl blocking reagents vary considerably in their capacity to inactivate the infectivity of viruses. It seems likely that such differential reactivity could be due to the degree of permeability of the alkylating reagents. To this end we compared the antiviral effect of highly permeable *N*-ethylmaleimide (NEM) with that of the impermeant analog, dextran-maleimide (Abbott and Schacter, 1976). In each case the capacity of these two reagents to compromise the infectivity of VSV was tested in the presence or absence of the reducing agent, 2-ME.

Table 1 illustrates the marked effect of NEM on the infectivity of VS virus; 1 mM NEM reduced plating efficiency of the virus by  $\sim 10^{-5}$ , whereas equivalent concentrations of IAA had no effect. No infectious virus could be detected after exposure to 10 mM NEM, a dose at which IAA had a barely detectable effect.

We next compared the effect of the im-

permeant, high molecular weight analog of maleimide, dextran-maleimide (MW  $\approx 10,000$ ), which Abbott and Schacter (1976) had shown efficiently labels surface sulfhydryl groups but does not penetrate the erythrocyte membrane. Table 1 shows that even 10 mM dextran-maleimide had little or no effect on the infectivity of VS virus; an equivalent concentration of the permeable maleimide reduces the plaque titer of VSV by at least  $10^{-7}$ . Even the presence of 2-ME did not result in significant decline of infectivity after exposure to dextran-maleimide.

These data appear to indicate that a highly diffusible sulfhydryl reagent can cause drastic loss in infectivity of VSV. Conversely, sulfhydryl blockers of low permeability are far less capable of inactivating virus infectivity; impermeable reagents have little or no effect even in the presence of a reducing agent. It seemed likely from these experiments that the major effect of sulfhydryl blocking reagents was on viral proteins internal to the limiting membrane rather than the external glycoprotein.

It was of interest to determine the degree to which iodoacetic acid could react with free sulfhydryl groups and reduced disulfides of proteins located internal to the limiting membrane of intact VSV, as well as those located on the external G protein. For this purpose, purified, intact virions were exposed to 50 mM [ $^{14}$ C]iodoacetic acid alone or following pretreatment with 50 mM 2-ME; free [ $^{14}$ C]iodoacetic acid was removed by overnight dialysis at 4° against 4000 vol of PBS. Proteins of unreduced and reduced virions were extracted by boiling in 2% SDS and analyzed by electrophoresis on 7.5% polyacrylamide gels.

The electropherograms shown in Fig. 1 compare the amount of [ $^{14}$ C]iodoacetic acid covalently linked to each protein of intact VS virions, unreduced or reduced by 2-ME. It can be seen that [ $^{14}$ C]iodoacetic acid penetrated the envelope of intact VSV and readily labeled free SH groups of all submembranous proteins, particularly M and N, even in the unreduced state. The presence of the reducing agent, 2-ME, resulted in greatly enhanced labeling by [ $^{14}$ C]iodoacetic acid of VSV proteins M, N, NS

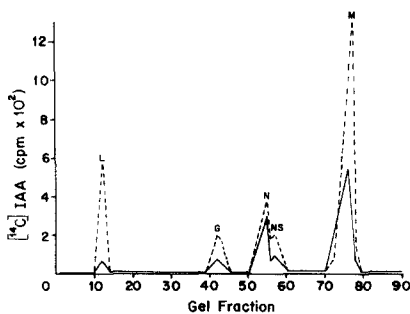


FIG. 1. Comparative electropherograms of proteins extracted from intact VSV that had been incubated with [ $^{14}\text{C}$ ]iodoacetic acid (200 mCi/mmol) (IAA) alone (solid line) or with [ $^{14}\text{C}$ ]IAA after prior reduction with 2-mercaptoethanol (dashed line). Purified, intact VSV (1 mg/ml  $\cong$   $4.25 \times 10^{12}$  particles/mg) was incubated for periods of 30 min at 25°C with 50 mM IAA alone or 50 mM 2-ME followed by 50 mM IAA. Viral proteins were extracted by boiling for 2 min in 2% SDS and separated by electrophoresis for 16 hr on 7.5% polyacrylamide gels at 4 mA/gel. The gels were sliced into 1-mm fractions and the radioactivity was determined by scintillation spectroscopy. The letters L, G, N, NS and M designated the peak positions of coelectrophoresed VSV  $^3\text{H}$ -protein markers.

and especially the L protein. Also shown in Fig. 1 is evidence for free SH groups in the external G protein spike as well as confirmation for the existence of disulfide bonds that could be labeled with [ $^{14}\text{C}$ ]iodoacetic acid after reduction by 2-ME. When VSV, unreduced or reduced with 2-ME, was disrupted by exposure to 2% Triton X-100, the proteins were labeled with [ $^{14}\text{C}$ ]iodoacetic acid to about the same extent as were the proteins of the intact virus (data not shown). These findings suggest that the VSV membrane does not serve to any great extent as a barrier to IAA or 2-ME.

The marked affinity noted above of iodoacetic acid for L protein, the putative VSV polymerase enzyme (Emerson and Wagner, 1973), led us to undertake to determine whether loss of VSV infectivity caused by sulfhydryl reagents could be attributed to their acting on the virion polymerase. Therefore, purified intact VSV was exposed to varying concentrations of SH-blocking reagents IAA or NEM, with or without prior exposure to 2-ME. After removal of excess reagents by centrifugation, control

and treated virus was disrupted in Triton-high salt solubilizer and polymerase activity was assayed by incorporation of [ $^3\text{H}$ ]uridine into acid-precipitable RNA (Emerson and Wagner, 1973).

Figure 2 shows that *in vitro* transcriptase activity of intact VSV was progres-

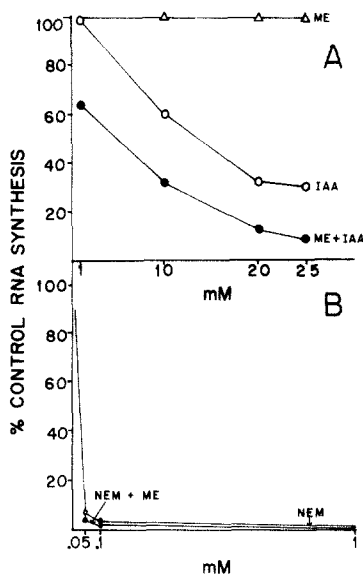


FIG. 2. Comparative inhibition of RNA synthesized by VSV after exposing intact virions to varying concentrations of (A) 2-mercaptoethanol alone (ME  $\Delta$ ), iodoacetic acid alone (IAA  $\circ$ ), or 2-mercaptoethanol + iodoacetic acid (ME + IAA  $\bullet$ ), or (B) *N*-ethylmaleimide alone (NEM  $\circ$ ) or *N*-ethylmaleimide + 2-mercaptoethanol (NEM + ME  $\bullet$ ). Purified intact virus was incubated for 30 min at 25° with buffer (controls) or with various concentrations of each of the reagents. Unreacted reagents were removed by pelleting the virus by centrifugation through a 50% glycerol cushion. As previously described (Hunt and Wagner, 1975), transcriptase activity of the virions was assayed by mixing 1 vol of virus suspension in RSB-15% glycerol with 1 vol of 2 $\times$  Triton-high salt solubilizer for 2 min at 0° and then by mixing with 3 vol of RSB-15% glycerol and 5 vol of prereaction mixture consisting of 1.3 mM dithiothreitol, 8 mM magnesium acetate, and 50 mM Tris-hydrochloride (pH 8.0) containing 1.4 mM each of ATP, GTP, CTP, and 0.133 mM [ $^3\text{H}$ ]UTP (230  $\mu\text{Ci}/\text{mmol}$ ). Reaction mixtures were incubated in stoppered tubes at 31° for 1 hr. Duplicate samples of 100  $\mu\text{l}$  each were precipitated with 10% trichloroacetic acid and the insoluble RNA was counted by scintillation spectroscopy. The results are expressed as percentage of the control polymerase reactions in the absence of inhibitory reagents.

sively inhibited by exposure to increasing concentrations of IAA and drastically inhibited by very small amounts of NEM. The effect of IAA on transcription was greater when intact virions were previously exposed to 2-ME, reaching <10% of the control level of RNA synthesis at 20–25 mM IAA (Fig. 2A). As expected, prior exposure to 2-ME alone had no effect on VSV transcription (Fig. 2A). In sharp contrast, 50  $\mu$ mol of NEM reduced VSV transcriptase activity at least 96%, regardless of whether 2-ME was present (Fig. 2B). These results indicate that sulfhydryl reagents inactivate transcriptase activity of intact VSV to varying degrees, potentially accounting for their capacity to reduce infectivity to a corresponding degree. Of considerable interest was the finding that the nonpenetrating sulfhydryl reagent, dextran-maleimide, had no capacity to reduce the transcriptase activity of intact VSV; however, 100  $\mu$ mol of dextran maleimide inhibited *in vitro* RNA synthesis of Triton-disrupted VSV by 99% (data not shown).

#### DISCUSSION

Earlier studies on the antiviral activity of sulfhydryl reagents implicated surface components of virions as the targets presumably by compromising cellular adsorption and/or penetration of the virus. Evidence that sulfhydryl reagents can affect capsid proteins or glycoproteins of enveloped virus was largely based on inhibition of hemagglutination by picornaviruses (Philipson and Choppin, 1962), paramyxoviruses (Scheid and Choppin, 1974), coronavirus (Pocock, 1978), and measles virus (Hardwick and Bussell, 1978). Our experiments reveal that potent sulfhydryl reagents have no effect on the hemagglutinating activity of vesicular stomatitis virus even though the free sulfhydryl groups and reduced disulfides of VSV glycoprotein are effectively blocked. Sulfhydryl reagents capable of penetrating the VSV membrane and reacting with internal proteins quite effectively impaired viral infectivity regardless of whether protein disulfide bonds were reduced. Con-

versely, the impermeant bulky reagent, dextran-maleimide, had no effect on the infectivity of VSV. The permeable sulfhydryl reagents, iodoacetic acid and *N*-ethyl-maleimide, inhibited the RNA polymerase of intact VSV, whereas dextran-maleimide did not unless the virion membrane was disrupted with Triton X-100. These data suggest that sulfhydryl reagents will inactivate the infectivity of intact VSV only if they are capable of penetrating the membrane and attack the endogenous RNA polymerase of the virion.

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