1973

Study of activities of vesicular stomatitis virus treated with 1-guanyl-3,5-dimethylpyrazole nitrate.

Richard A. Szabo
University of Windsor

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STUDY OF

ACTIVITIES OF VESICULAR STOMATITIS VIRUS TREATED

WITH 1-GUANYL-3,5-DIMETHYLPYRAZOLE NITRATE

by

Richard A. Szabo

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

WINDSOR, ONTARIO, CANADA

1973
ABSTRACT

The modification of proteins by 1-quanyl-3,5-dimethylpyrazole nitrate (GDMP) has been reported. In the present investigation this reagent was found to be inhibitory to vesicular stomatitis (VS) virus infectivity. When VS virus was treated with GDMP a reduced sensitivity to the virucidal drug, Kethoxal, was shown as well as increased neutralizability by VS specific calf antiserum and altered electrophoretic profiles of SDS-DTT treated virus.

Virus grown in different host cells were similar to L cell-grown virus in that GDMP treatment caused diminished sensitivity to the drug.

Virus harvested early from GDMP-treated L cells was also characterized by a decreased sensitivity to Kethoxal, but this effect was not evident in late harvested viral progeny.
ACKNOWLEDGMENTS

The author wishes to express sincere gratitude to Dr. L. R. Sabina, Department of Biology, University of Windsor, for his foresight, guidance and encouragement throughout the course of this research.

The author is grateful also to Dr. A. H. Warner, Department of Biology and Dr. R. J. Thibert, Department of Chemistry, both of the University of Windsor, for their aid in the reviewing of this thesis.

A note of thanks is extended to Messrs. F. M. Lukacs and T. D. Edwards for their tolerant assistance in providing research materials.

For patience, understanding, and inspiration from those closest, a wholehearted thank you will barely suffice.

This investigation was supported by grant A 2904 from the National Research Council of Canada.
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INTRODUCTION

The concept of chemical modification of drugs for the purpose of conferring a wider antiviral spectrum is well established. For example, a number of derivatives of isatin-β-thiosemicarbazone (Bauer and Sadler, 1960; Pearson and Zimmerman, 1969) and ribosides of purine and pyrimidine bases (Underwood et al., 1964; Cohen, 1966; Schabel, 1968) have been given considerable attention concerning their capacity to inhibit virus replication.

An alternate approach to arrest viral replication involves modification of the virion itself. In the pharmaceutical industry, application of this idea led to the use of formalin, aluminum compounds, β-propiolactone, etc., to modify viruses. Such chemical treatments result in detoxified virions which are incapable of replication, yet retain their ability to elicit an immunogenic response in vivo.
Some commonly used procedures for chemical modification of proteins include acetylation, succinylation, sulfonation, and guanidination. Because most of these procedures incorporate relatively harsh conditions (Means and Feeney, 1971), the chemical modification of viruses may result in the loss of certain biological activities associated with surface structures. Choppin and Philipson (1961) found p-chloromercuribenzoate inactivates human enterovirus infectivity by altering surface attachment sites. Later, Kunin (1967) reported that encephalomyocarditis virus infectivity was also destroyed by exposure to p-mercuribenzoate, and by periodate oxidation and acetylation as well.

There is limited information in the literature, however, regarding the alteration of surface morphology of different strains of enveloped RNA viruses by use of proteases. Reginster (1965) was able to modify influenza type A (PR8) virus with caseinase C. He found that the hemagglutinating
activity and infectivity of the virus were greatly reduced whereas neuraminidase activity was unchanged. Biddle (1968) demonstrated that the action of protease on Asian influenza (A₂) results in the destruction of V antigen and hemagglutinin but the viral preparation had neuraminidase activity and some infectivity. More recently, Morrow (1972) showed that vesicular stomatitis virus subjected to various proteolytic enzymes was less refractory to the antiviral, Kethoxal.

The present study was undertaken to investigate whether surface proteins of VS virus could be chemically modified without considerable loss of infectivity. Guanidination of proteins under mild conditions has been reported to have little effect on the physical and chemical properties of proteins which are stable in strongly alkaline solution (Means and Feeney, 1971). Since VS virus infectivity is not destroyed until the level of pH exceeds 11.6 (Fong and Madin, 1954), we expected that the product resulting from guanidination
of virus would be infective. Because this expectation has been borne out, the study was also aimed at obtaining data on the degree of sensitivity of guanidinated vesicular stomatitis surfaces to the virucidal action of Kethoxal.

In this study, VS virus, a member of the group of rhabdoviruses which have a highly characteristic bullet-like shape was used. The virus contains RNA and measures 175 x 65 nm. It possesses a rigid helical internal framework which is considered to be the nucleoprotein component and its exact conformation remains unresolved (Cartwright et al., 1972). A tightly fitting envelope encloses the bullet-shaped shell. In addition, the virus exhibits spike-like projections composed of protein (Howatson and Whitmore, 1962) which are fundamental to its infectivity (Cartwright et al., 1968).
MATERIALS AND METHODS

Cells and Media

The following heteroploid cell lines were used in the experiments: MDBK cells of bovine kidney origin and L-929 cells derived from a mouse fibrosarcoma were obtained from the American Type Culture Collection Cell Repository, Rockville, Maryland; and MA-104 cells from Rhesus monkey kidney, FL cells from normal human amnion tissue and H.Ep. #2 cells from a human carcinoma of the larynx were supplied by Microbiological Associates, Inc., Bethesda Maryland.

Monolayer cultures of MDBK, MA-104, and H.Ep. #2 were grown and maintained in Eagles' (1959) minimal essential medium (MEM) containing 10% fetal calf serum. In addition, MEM was supplemented with antibiotics (penicillin 100 units/ml, streptomycin 100µg/ml, mycostatin 25µg/ml). FL and L cell monolayers were propagated and maintained in medium ELAY-10FCS as previously described (Sabina and Parker, 1963).
Virus

Vesicular stomatitis (VS) virus, Indiana serotype, was obtained from Dr. N. A. Labzofsky, Ontario Department of Health, Toronto, Ontario. A single virus seed stock was prepared by inoculating MDBK monolayers at an input of 0.8 to 1 plaque forming unit (PFU) per cell. The infectious cultures at 30 hours postinfection were frozen and thawed twice. After removal of cellular debris by centrifugation for 10 minutes at 1100 x g (Sorvall RC-2B; Rotor no. SS-34), the virus-containing culture fluids were combined then dispensed in aliquots for storage at -60°C. The virus seed stock was used to prepare pools of virus in L-929 cells by the method described above for all experiments unless otherwise stated. These virus pools had infectivity titers ranging from $2 \times 10^7$ to $3.2 \times 10^8$ PFU/ml.

Virus purification

For semipurification of VS virus an aliquot of the virus
pool was centrifuged for 2 hours at 42,500 x g (Beckman Model L2-65B; Rotor no. 60 Ti). The sedimented viruses were resuspended in a small volume of either Dulbecco's phosphate-buffered saline (PBS) minus magnesium and calcium salts, pH 7.4 (Merchant et al., 1964) or 0.0125 M sodium borate-sodium hydroxide buffer (BH). pH 10. In some experiments the virus suspension was further purified by centrifugation through a preformed discontinuous type of sucrose gradient (see Appendix A) for 2 hours at 92,500 x g in a Beckman SW 41 Ti rotor. The strongly opalescent region (band III) which sedimented atop the 45% sucrose layer, was found to contain most of the infectious virus. Such infectious virus bands had about 30μg of protein/ml when estimated by the method of Lowry et al. (1951).

Infectivity assays

Infectious virus was assayed by plaque counting in L cell monolayers as previously reported (Sabina and Munro, 1969).
Chemicals

Aminoguanidine nitrate and 2,4-pentanedione were purchased from Eastman Organic Chemicals, Rochester, New York; sodium dodecyl sulfate (SDS) and sodium borohydride from Fisher Scientific Company, Fair Lawn, New Jersey; and dithiothreitol (DTT) from Calbiochem, San Diego, California. 1-Guany1-3,5-dimethyl pyrazole nitrate (GDMP) was synthesized according to the procedure of Bannard et al. (1958). Kethoxal (2-keto-3 ethoxy butyraldehyde hydrate) was made available by Dr. G. E. Underwood, the Upjohn Company, Kalamazoo, Michigan.

Reaction of GDMP with VS virus

Semipurified viruses resuspended in small volumes of 0.0125 M BH buffer, pH 10 were mixed with equal volumes of GDMP dissolved in the same buffer at concentration levels ranging from 0.0038 to 0.28 M. The mixtures were magnetically stirred in the dark at room temperature for 60 minutes and
were then chilled in an ice-bath. Control virus preparations were subjected to similar conditions as the experimental samples. GDMP-treated virus and control virus mixtures were layered on discontinuous sucrose gradients and centrifuged under conditions previously described. After centrifugation, band III (unless otherwise stated) from each sucrose gradient was collected for infectivity assay and for in vitro virucidal tests.

**Treatment of L cells with GDMP**

Monolayers of L cells in 4-oz. Brockway bottles were washed with PBS buffer and virus was added at an input multiplicity of about 2 PFU per cell. An adsorption period of 1 hour at 37°C was allowed before the media containing residual virus was removed from the bottles. The monolayers were washed, then overlaid with fresh medium and again incubated at 37°C for 1 hour. At 2 hours postinfection time the cultures were washed twice with 0.15 M NaCl solution containing 5 x 10^{-5} M
\[ \text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}, \text{pH } 10, \text{ (NaCl-BH)} \text{ and divided into 2 groups.} \]

One group of cultures was overlaid with 0.0038 M GDMP in BH buffer and the other group received only NaCl-BH solution.

All cultures were drained of overlay solution after incubation for 2 hours at 37°C. These cultures were rinsed and refed with medium ELAY - 10FCS and reincubated at 37°C for appropriate period before harvesting infectious virus.

**In vitro virucidal assay of Kethoxal**

One part of purified virus was mixed with one part of Kethoxal previously diluted in PBS solution to give the appropriate concentration under test. The mixture was incubated in the dark either at room temperature for 30 minutes or at 37°C for 15 minutes and then assayed for infectious virus.

In the case of GDMP-treated virus, the chemical-virus mixture was centrifuged through a discontinuous sucrose gradient. Thus excess GDMP is removed and band III extracted
for treatment with Kethoxal as described above. By using the
infectivity values for GDMP-treated virus + Kethoxal (A),
GDMP-treated virus (B), and untreated virus (C), the per cent
infectivity reduction can be calculated as follows:

\[
\frac{A - B}{C} \times 100 = \% \text{ Infectivity reduction.}
\]

**SDS - Polyacrylamide gel electrophoresis**

SDS - Polyacrylamide gel electrophoresis was performed on
GDMP-treated and untreated virus preparations according to the
method of Reisfield and Small (1966) with the modifications of
Raam and Inman (1973). To obtain sucrose-free viral preparations,
the collected samples (band III) were diluted in 0.0125 M BH
buffer, pH 10 and recentrifuged at 50,000 x g for 2 hours. The
virus pellets were solubilized in a solution containing 1% SDS,
0.1 M dithiothreitol, 0.32 M NaCl, 0.1 M Tris, pH 8 by heating
at 100°C for 1 minute. Samples containing approximately 50μg
of protein were mixed with marker dye (bromphenol blue) and
applied to the 4% polyacrylamide gels in a Shandon Disc
Electrophoresis Apparatus. These gels were subjected to electrophoresis at 4 mA per gel for 35 minutes at room temperature. The gel columns were stained with Amido black, cleared and densitometric tracings of the stained gels were made.

**Measurement of serum neutralizing activity.**

Calf antiserum directed against the Indiana serotype of vesicular stomatitis virus was kindly provided by Dr. N. G. Willis, Animal Diseases Research Institute, Hull, Quebec.

The virus-neutralizing activity of the hyperimmune serum was measured by mixing equal volumes of purified virus and antiserum diluted 1:10 in PBS. Mixtures were maintained at 37°C. Appropriate samples were taken at various intervals and assayed by plaque counting on L cell monolayers for the amount of surviving virus.
EXPERIMENTAL RESULTS

The effect of virucidal conditions on yields of VS virus reacted with various levels of Kethoxal

To establish the sensitivity of VS virus to Kethoxal under different reaction conditions, purified virus preparations were mixed with varying concentrations of drug as described in Materials And Methods and assayed for infectious virus.

When viruses were suspended in increasing concentrations of Kethoxal, there was a marked decrease in viral yields (Table 1). It was found that virus yields declined at different rates such that virus suspended in 0.1 µg/ml of Kethoxal resulted in about a 17% loss at room temperature for 30 minutes while at 37°C for 15 minutes, a decline of 58% occurred. Irrespective of the temperature-time conditions used in the virucidal test, 20 µg/ml of drug caused a reduction in virus yield greater than 99%. Because the virucidal activity of Kethoxal against
Table 1. The effect of virucidal conditions on yields of VS virus reacted with various levels of Kethoxal.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Concentration of Kethoxal (µg/ml)</th>
<th>Viral yields (PFU/ml)</th>
<th>Per Cent survival</th>
<th>Viral yields (PFU/ml)</th>
<th>Per Cent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>$7.4 \times 10^6$</td>
<td>100.0</td>
<td>$7.5 \times 10^6$</td>
<td>100.0</td>
</tr>
<tr>
<td>0.1</td>
<td>$3.1 \times 10^6$</td>
<td>41.8</td>
<td>$6.2 \times 10^6$</td>
<td>82.7</td>
</tr>
<tr>
<td>0.5</td>
<td>$2.5 \times 10^6$</td>
<td>33.8</td>
<td>$5.8 \times 10^6$</td>
<td>77.3</td>
</tr>
<tr>
<td>0.6</td>
<td>$4.6 \times 10^5$</td>
<td>6.2</td>
<td>$5.0 \times 10^6$</td>
<td>66.7</td>
</tr>
<tr>
<td>10.0</td>
<td>$4.8 \times 10^4$</td>
<td>0.6</td>
<td>$5.1 \times 10^5$</td>
<td>6.8</td>
</tr>
<tr>
<td>20.0</td>
<td>$6.5 \times 10^2$</td>
<td>&lt;0.1</td>
<td>$7.2 \times 10^3$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>50.0</td>
<td>&lt;10\textsuperscript{1}</td>
<td>&lt;0.1</td>
<td>1.1 \times 10\textsuperscript{2}</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Kethoxal was diluted in PBS, pH 7.4.

\textsuperscript{b} Room temperature.
virus was more rapid at 37°C than that at room temperature and because of possible fluctuations in room temperature from one experiment to another, all subsequent virucidal testing was carried out at 37°C for 15 minutes.

The effect of GDMP treatment on virus yields

Since moderately alkaline conditions are required for the guanidination of proteins, it was important to know whether BH buffer, pH 10 would influence virus infectivity. Although not shown here, when semipurified virus was resuspended in BH, pH 10 or PBS, pH 10 and then assayed for infectivity there was a comparable yield of virus produced. However, a 33% decrease was demonstrated when compared to the yield obtained with PBS, pH 7.4. From this experiment it was apparent that borate was not toxic but pH did influence yields of virus.

In view of the preceding experiment it seemed that an adequate number of infectious virus was left for reaction
Figure 1. The relationship between GDMP concentration and virus infectivity.
with GDMP. Figure 1 shows the relationship between GDMP concentrations and virus infectivity. In the presence of 0.0038 M GDMP virus infectivity was reduced about 60% but increasing the concentration to 0.06 M had a drastic effect on infectivity.

In an attempt to stabilize the product arising from guanidination, sodium borohydride (NaBH$_4$) solution was added to the virus-GDMP reaction mixture. At a concentration of 0.02 M, NaBH$_4$ was shown to effectively inactivate virus infectivity (data omitted).

The effect of GDMP treatment on virus sensitivity to Kethoxal

To quantitate the sensitivity of GDMP-treated and untreated VS virus to Kethoxal, the drug was applied to virus preparations at levels of 0.1 and 0.5 µg/ml for the virucidal assay. The amount of infectivity reduction was expressed as a percentage of the titer obtained with untreated controls as described under Materials And Methods. The results are
summarized in Table 2. The extent of sensitivity of untreated and GDMP-treated virus to Kethoxal showed that the latter was considerably less sensitive when tested against different drug levels. GDMP-treated virus was relatively unaffected by Kethoxal amounting to 1 to 5% loss of infectivity. With untreated virus about 57 to 67% of the infectious virus was inactivated by the drug.

Virucidal activity of Kethoxal against GDMP-treated and untreated virus separated on discontinuous sucrose gradients.

Because defective virions of different molecular size are commonly synthesized during VS virus infections, discontinuous sucrose gradients were used to isolate the infectious progeny for virucidal assay. Figure 2 represents a typical discontinuous gradient with the various concentration steps of sucrose employed and the approximate location of virus bands. After centrifugation of virus through a gradient, 3 distinct bands and a pellet were observed in both GDMP-treated
<table>
<thead>
<tr>
<th>Test virus</th>
<th>Conc. of Kethoxal (µg/ml)</th>
<th>Infectivity (PFU/ml)</th>
<th>% Infectivity reduction</th>
<th>Infectivity (PFU/ml)</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS</td>
<td>0.0</td>
<td>$1.4 \times 10^6$</td>
<td>-</td>
<td>$1.3 \times 10^6$</td>
<td>-</td>
</tr>
<tr>
<td>VS</td>
<td>0.1</td>
<td>$5.4 \times 10^5$</td>
<td>61.4</td>
<td>$5.6 \times 10^5$</td>
<td>56.9</td>
</tr>
<tr>
<td>VS</td>
<td>0.5</td>
<td>$4.7 \times 10^5$</td>
<td>66.4</td>
<td>$4.4 \times 10^5$</td>
<td>66.2</td>
</tr>
<tr>
<td>VS-GDMP</td>
<td>0.0</td>
<td>$5.0 \times 10^5$</td>
<td>-</td>
<td>$5.0 \times 10^5$</td>
<td>-</td>
</tr>
<tr>
<td>VS-GDMP</td>
<td>0.1</td>
<td>$4.9 \times 10^5$</td>
<td>0.7</td>
<td>$4.8 \times 10^5$</td>
<td>1.5</td>
</tr>
<tr>
<td>VS-GDMP</td>
<td>0.5</td>
<td>$4.5 \times 10^5$</td>
<td>3.5</td>
<td>$4.4 \times 10^5$</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\( ^a \) Experimental conditions as described under Materials and Methods. GDMP was used at a concentration of 0.0038 M.
Figure 2. A typical discontinuous gradient with various sucrose concentrations. Infectious virus was primarily associated with Band III.
Virus band

Initial % sucrose

I
II
III
pellet
and untreated virus preparations. Generally a wider band III was obtained with GDMP-treated virus when compared with controls.

It is evident (Table 3) that infectivity was associated with all virus bands. Although not shown, the pellets of GDMP-treated and untreated virus had infectivities of \(4 \times 10^6\) and \(3.4 \times 10^6\) PFU/ml, respectively. Therefore, at least 85% of the total infectious virus was recovered from band III.

The data in Table 3 indicates that the sensitivity to Kethoxal of band III virus treated with GDMP was markedly depressed. By comparison however, GDMP-treated virus bands I and II displayed less pronounced depression of sensitivity to the drug. The pellet was not tested for sensitivity to Kethoxal because of the possible presence of highly aggregated virus. Virus recovered from band III was used in all investigations unless otherwise stated.

The influence of cell line on progeny virus sensitivity to Kethoxal

To study the influence of host cell on progeny virus
Table 3. Virucidal activity of Kethoxal against GDMP-treated and untreated virus separated on discontinuous sucrose gradients.

<table>
<thead>
<tr>
<th>Conc. of Kethoxal (μg/ml)</th>
<th>Virus yields</th>
<th>Infectivity reduction</th>
<th>Virus yields</th>
<th>Infectivity reduction</th>
<th>Virus yields</th>
<th>Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5.2 x 10^5</td>
<td>--</td>
<td>2.0 x 10^6</td>
<td>--</td>
<td>8.0 x 10^7</td>
<td>--</td>
</tr>
<tr>
<td>0.1</td>
<td>5.5 x 10^4</td>
<td>89.4</td>
<td>1.3 x 10^6</td>
<td>35.0</td>
<td>3.5 x 10^7</td>
<td>56.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0 x 10^4</td>
<td>96.2</td>
<td>2.8 x 10^5</td>
<td>85.8</td>
<td>1.6 x 10^6</td>
<td>98.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GDMP-treated virus</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5.2 x 10^5</td>
<td>--</td>
<td>2.0 x 10^6</td>
<td>--</td>
<td>3.6 x 10^7</td>
<td>--</td>
</tr>
<tr>
<td>0.1</td>
<td>3.9 x 10^5</td>
<td>25.0</td>
<td>1.9 x 10^6</td>
<td>6.5</td>
<td>3.4 x 10^7</td>
<td>2.5</td>
</tr>
<tr>
<td>0.5</td>
<td>1.6 x 10^5</td>
<td>68.8</td>
<td>7.1 x 10^5</td>
<td>64.6</td>
<td>5.6 x 10^6</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Experimental design as for Table 2, except all sucrose gradient bands were used for the virucidal assay.
sensitivity to Ketoxal, seed virus (MDBK host) was passaged 3 times in FL, H.Ep. #2 or MA-104 cell culture systems.

Pools of each were prepared and the virucidal activity of Ketoxal against GDMP-treated and untreated virus was examined. Although H.Ep. #2 cells yielded substantially more virus than cell lines FL and MA-104, the amount produced was only about 8% of that in MDBK cells (Table 4). No matter which cell line was used the sensitivity to Ketoxal was demonstrated to be reduced by GDMP treatment. The degree of sensitivity, however, was somewhat variable.

Virucidal assay of virus harvested from GDMP-treated L cells

It was established that L cell monolayers pre-treated for 2 hours with NaCl-BH solution before virus addition yield high-titered VS virus. In view of this finding it was possible to investigate whether virus harvested from cells overlaid with alkaline GDMP would reflect altered sensitivity to Ketoxal. To evaluate the sensitivity of progeny virus to
Table 4. The influence of cell line on progeny virus sensitivity to Kethoxal.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Conc. of Kethoxal (µg/ml)</th>
<th>Virus yields (PFU/ml)</th>
<th>% Infectivity reduction</th>
<th>Virus yields (PFU/ml)</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td></td>
<td>GEMP-treated</td>
<td></td>
</tr>
<tr>
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\textsuperscript{a} Virus stocks were prepared in different cell lines as in Materials and Methods and the experimental design was as for Table 2.
Kethoxal, monolayers of L cells were infected with virus, treated with GDMP and virus progeny harvested at 12, 22, and 32 hours postinfection for the virucidal assay as described in Materials And Methods. Kethoxal sensitivity of progeny virus harvested from GDMP-treated cells at 12 and 22 hours postinfection was reduced about 20 to 34% when compared with controls. Surprisingly, the 32 hour progeny from GDMP-treated and untreated cells were equally sensitive to the drug. A comparison of Table 5 with Table 2 indicates that greater reduction in sensitivity to Kethoxal is attainable by treating virus rather than host cells with GDMP.

Serum neutralization of GDMP-treated VS virus

GDMP-treated and control virus preparations were incubated with VS-antiserum as described in order to assess whether they were immunologically distinguishable. As indicated in Figure 3, the rate of neutralizability of virus was altered considerably when treated with GDMP. By 7.5
Table 5. Virucidal assay of virus harvested from GDMP-treated L cells.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Time of Harvest (hr)</th>
<th>Conc. of Kethoxal (μg/ml)</th>
<th>Virus yields (PFU/ml)</th>
<th>% Infectivity reduction</th>
<th>Virus yields (PFU/ml)</th>
<th>% Infectivity reduction</th>
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\textsuperscript{a} L cells were treated with 0.0038 M GDMP from 2 to 4 hours postinfection with VS virus. Virus was harvested, and purified for virucidal assay.
Figure 3. The rate of neutralization of VS virus by specific antiserum. Symbols: GDMP-treated (O), control (●).
minutes about 82% of GDMP-treated virus was neutralized whereas control virus was inactivated only 18%. From this experiment it appears that GDMP treatment blocked infectivity sites on the viral surface since a lesser number of antibodies were needed for neutralizing virus.

**Comparison of GDMP-treated and untreated VS-virus proteins**

For this investigation GDMP-treated virus and virus prepared in PBS, pH 7.4 and BH, pH 10 were solubilized in SDS and subjected to electrophoresis on polyacrylamide gels. Figure 4 represents densitometric tracings of SDS-polyacrylamide gels of stained VS virus proteins. The three profiles were somewhat similar. Although 5 different protein bands were obtained, some bands were not clearly resolved. The three lightest viral proteins of control virus, pH 10 migrated slightly faster than those of GDMP-treated virus. Electrophoresis of virus protein in PBS, pH 7.4 showed that band 2 was more sharply resolved when compared with the other
Figure 4. A diagramatic representation of densitometric tracings of VS virus proteins in SDS-polyacrylamide gels.

Purified virus preparations in PBS, pH 7.4 (A), BH, pH 10 (B) and GDMP solution, pH 10 (C) were solubilized and electrophoresed as in Materials And Methods.
two virus preparations.
DISCUSSION

The guanidination of VS virus has been studied with respect to its effect on viral infectivity, the virucidal action of Kethoxal and antigenic sites. Other studies (Habib, 1960; Spero, 1971) have established that 1-guanyl-3,5-dimethylpyrazole nitrate (GDMP) can be used to effectively guanidinate biological proteins under mild alkaline conditions without loss of activity. The compound reacts with \( \alpha \) - and \( \epsilon \) -amino groups of proteins at pH 9.5 by replacing them with more basic guanidino groups. The structure of GDMP is shown below:

![Chemical structure of GDMP](image)
Treatment of VS virus with increasing concentrations of GDMP had a drastic effect on viral yields. Only about 40% infectious virus remained when treated with 0.0038 M GDMP. This finding was not surprising since infectivity sites of enveloped viruses are more sensitive to inactivation than other surface structures (Schlotissek and Rott, 1963). However, the residual virus was found to be neutralizable with VS specific antiserum. Since GDMP-treated virus was more readily neutralized than control virus by antiserum, it suggests that infectivity sites are part of some of the antigenic surface structures. Perhaps the presence of one or more guanidino groups in the antigenic structures produce a distorted configuration of the viral coat which enhances neutralizability. It is also possible that amino groups in the infectivity site and/or antigenic region differ in their capacity to bind guanidino groups. Therefore blockage of amino acid residues which are not a critical part of the
antigenic determinant may facilitate neutralization.

The ability of Kethoxal to inactivate GDMP-treated VS virus is strikingly diminished as compared to untreated controls. It is feasible that blockage of Kethoxal reaction groups occurs on the viral surface. Underwood et al. (1959) determined that Kethoxal reacts with α-amino groups. In addition, they suggest the guanidino grouping as another site of reaction with Kethoxal. If both α- and ε-amino groups on the viral coat are blocked by guanidino groups, perhaps more reaction sites are now available to Kethoxal and it is binding in areas not critical to viral infectivity, when used in low concentrations.

This diminished drug sensitivity is observable although to different degrees when VS virus is harvested from different host cell lines. Apparently, GDMP still reacts with the viral coat but because of different results in virucidal assays it seems that the viral protein coat may have slight
differences in make-up as evidenced by Cartwright, (1968). He suggested that VS virus receives coat protein material from the host cell membrane.

When VS infected L cells were treated with GDMP, early progeny showed a striking decrease in drug sensitivity. The virus during replication may have incorporated guanidinated host protein into its own coat. Since late progeny did not show this effect it is possible that guanidinated proteins have already been incorporated into virus or that by this time GDMP has been eliminated from the host membrane.

The data indicated that electrophoretic mobilities of GDMP-treated virus proteins were slightly altered. The treated virus was less electronegative than untreated controls. In addition to charge differences, the replacement of amino groups by the sterically different guanidino groups may have played an important role in retarding electrophoretic migration of the proteins.
In view of these findings it appears that chemical modification of the viral surface of vesicular stomatitis, without complete loss of infectivity, is possible.
SUMMARY

The effect of guanidination of VS virus on its biological activity has been investigated. It was established that treatment of virus in band III with low levels of the compound caused some loss in infectivity; the remaining infective viral particles showed a marked depression in sensitivity to low concentrations of the virucidal drug, Kethoxal. A similar but less pronounced effect was created on virus in bands I and II.

Virus produced in FL, H.Ep. #2, MA-104, MDBK cell lines when treated with GDMP showed reduced sensitivity to Kethoxal. The degree of reduction, however, was somewhat variable.

Virus harvested at different times from L cells overlaid with GDMP solution from 2 to 4 hours postinfection varied in Kethoxal sensitivity.

GDMP-treated virus is apparently more readily neutralized
by VS virus directed calf antiserum than untreated virus.

Proteins of GDMP-treated virus showed different electrophoretic mobilities than those of the native virions.
APPENDIX A

Preparation of Discontinuous Gradients

Discontinuous gradient columns were prepared by floating layers of 1.5, 1.5, 4.0 and 2.0 ml of solutions of 50, 200, 300 and 450 mg sucrose/ml in 9/16 x 3 1/2 inch nitrocellulose centrifuge tubes. Ribonuclease - free grade sucrose was dissolved in sterile phosphate - buffered saline, pH 7.4. Before use the layered sucrose solutions were allowed to stand 8 hours at 4°C.
REFERENCES


UNDERWOOD, G. E., C. A. Wisner and S. D. Weed. 1964. Cytosine arabinoside (CA) and other nucleosides in herpes-virus infections. Laboratory Sciences, 72, 505-512.
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