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The antiviral activity of 2-keto-3-ethoxy butyraldehyde (Kethoxal) against vesicular stomatitis virus.

D. Gail. Morrow

University of Windsor

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THE ANTIVIRAL ACTIVITY OF
2-KETO-3-ETHOXY-BUTYRALDEHYDE (KETHOXAL)
AGAINST VESICULAR STOMATITIS VIRUS

by

D. Gail Morrow

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

1972
ABSTRACT

The in vitro virucidal activity of Kethoxal against a number of DNA and RNA viruses has been reported. In the present study, Kethoxal was shown to possess two modes of antiviral action against the RNA virus, vesicular stomatitis (VS) virus, including inhibition of intracellular virus multiplication and direct virucidal action on extracellular virus. When VS virus was pretreated with proteolytic enzymes, namely fungal and bacterial proteases, α- and β-chymotrypsins and trypsin, the antiviral activity of the drug was considerably reduced. The infectivity of Kethoxal-treated VS virus could not be regenerated by trypsin treatment. Exposure to β-chymotrypsin, RNase, low levels of phospholipase C or the chemical agents, Tween 20 or NH₂OH.HCl, did not alter VS virus sensitivity to Kethoxal. The results provide suggestive evidence that Kethoxal binding sites are available on the envelope or substructure of VS virions.
ACKNOWLEDGEMENTS

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

The author is grateful also to Dr. W. G. Benedict, 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.

To Messrs. F. M. Lukacs and T. D. Edwards a special thanks is extended for their assistance with research materials.

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INTRODUCTION

During the last two decades the search for suitable antiviral agents has been of primary importance in the chemotherapeutic industry.

Even though most antiviral agents have demonstrated outstanding in vitro activity against human viruses, only a few agents have so far proved effective in vivo (Hornick et al., 1970; Tomlinson & MacCallum, 1970; Turner et al., 1962). Moreover, antiviral chemotherapeutic agents effective in vivo must possess selective toxicity before they merit clinical trials in man (Penner & White, 1970). From these points of view at least six antiviral agents have been reported to be clinically useful in the prophylaxis and treatment of virus infections: N-methyl-isatin B-thiosemicarbazone (Marboran); 5-ido-2'-deoxyuridine (IDU); 9-β-D-arabinofuranosyl adenine (ara-A); 1-adamantanamine hydrochloride (amantadine); and 2-keto-3-ethoxy butyraldehyde hydrate (Kethoxal).

McLimans and coworkers (1957) found Kethoxal and closely related
compounds with a terminal \( \alpha \) - ketoaldehyde or \( \alpha \) - hydroxyaldehyde grouping are active against influenza A (strain PR - 8) and Newcastle disease (strain NJ - KD) viruses in embryonated eggs. Their data shows that Kethoxal is active when given 24 hours prior to inoculation or 6 hours after inoculation with virus. This observation suggests a direct virucidal action of the drug on extracellular virus. Underwood (1956) found Kethoxal activity to be questionable when tested against RNA viruses in chickens, ferrets and mice. In this investigation, low level antiviral activity or inconsistent results were obtained with the drug. Later, Underwood et al. (1959) concluded that the lack of in vivo activity of Kethoxal could be attributed to its inactivation by rapid binding to metabolites in the complex animal system. However, Kethoxal is highly active when applied topically to cutaneous herpes simplex lesions in both baby rabbits and hairless mice (Underwood, 1963). In clinical studies (Underwood and Nichol, 1971) Kethoxal activity was found to be considerably less effective against cutaneous herpes lesions in human volunteers than in laboratory animals. Kethoxal has been
shown to possess varying carcinostatic activity in vivo. For example, French and Freedlander (1958) demonstrated that the drug increases the life span of mice with Leukemia L 1210 and Furst et al. (1957) reported the drug to be highly active against Ehrlich Hauschka ES ascites tumor.

In a survey of the in vitro virucidal activity of Kethoxal, Underwood and Weed (1961) reported complete inactivation of mumps, influenza A and B, measles, Newcastle disease, Western equine encephalitis, vaccinia, B - virus, adeno and mouse hepatitis viruses. However, the virulent MEF - 1 strain and the attenuated strain of type 2 poliovirus were refractory to the antiviral. It also was found that Kethoxal has no virucidal effect on the three types of poliovirus and Coxsackie A - 21 which are RNA containing viruses (Renis, 1970). Yet under single-cycle conditions, the multiplication of Coxsackie A - 21 was inhibited when the drug was incorporated in the virus growth medium. Further investigation showed that after removal of the virus capsid of Coxsackie A - 21, the infectious viral RNA was susceptible to the virucidal effect of Kethoxal. Similarly, when infectious RNA is
isolated from tobacco mosaic virus it can be readily inactivated upon exposure to Kethoxal (Stahlherr, 1959).

Because Kethoxal is one of the few antiviral agents that has been found acceptable for clinical trials in man and because of its potent virucidal effect on vesicular stomatitis (VS) virus (Henss, 1970), it seemed desirable to use VS virus as an experimental model to examine additional in vitro activities of this drug. VS virus, a member of the group of bullet-shaped viruses, is a large RNA-containing virus measuring 175 x 65 nm. It possesses a rigid helical internal framework which is considered to be the nucleoprotein component, although its exact conformation is unresolved (Cartwright et al., 1969). An interesting feature of this virus is the presence of spike-like surface projections (Howatson & Whitmore, 1962). It was thought that modification of the virion surface through the use of enzymes and chemical agents would provide additional information concerning the antiviral activity of Kethoxal.
MATERIALS AND METHODS

Cells and Media

The bovine kidney cell line designated MDBK and the L cell line (NCTC clone 929) of murine origin were obtained from the American Type Culture Collection Cell Repository, Rockville, Maryland. These cells were grown as monolayers in medium ELAY - 10 FCS as previously described (Sabina & Parker, 1963) and maintained in medium ELAY - 5 FCS. For the experiments designed to study VS virus growth in the presence of Kethoxal, Eagle's synthetic minimal essential medium (MEM) (Eagle, 1959) was used with or without the addition of Kethoxal.

Virus

The Indiana serotype of vesicular stomatitis (VS) virus, provided by Dr. N. A. Labzoffsky, Ontario Department of Health, Toronto, Ontario, was used in this study. The virus was plaque purified twice in L cells. Virus stock was prepared by inoculating L cell monolayers with plaque purified virus at a multiplicity of 0.2 plaque forming units (PFU) per
cell. After adsorption at 37°C for 45 minutes, medium E1AY - 10 FCS was added and the cultures were reincubated. At 36 hours postinfection, pools of virus were obtained by subjecting the infectious cultures to 3 cycles of freezing and thawing and then the sediments were separated by centrifugation for 10 minutes at 1100 × g (Sorvall RC - 2B; Rotor no. SS - 34). The supernatant fluids were pooled and stored at -60°C. These stocks had infectivity titers ranging from 5.8 × 10^6 to 7.8 × 10^7 PFU/ml.

**Virus Assay**

VS virus was assayed by plaque formation in monolayers of either MDBK cells or L cells grown in 60-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, California). Dilutions of the samples were made in medium MEM supplemented with 10% fetal calf serum. One half ml aliquots of appropriate dilutions were allowed to adsorb at 37°C for 45 minutes. The cultures were then overlaid with 5 ml of agar medium.
(Sabina & Munro, 1969), and the plates were incubated at 37°C in a water saturated atmosphere of 5% CO₂ in air. The plaques were counted after approximately 24 hours.

**Enzymes, Chemicals and Antiserum**

The following were obtained from Sigma Chemical Company, St. Louis, Missouri: trypsin (2x crystallized), phospholipase C (from *Clostridium welchii*), ribonuclease A (5x crystallized), protease (from *Streptomyces griseus*), γ-chymotrypsin (2x crystallized), β-chymotrypsin (1x crystallized), α-chymotrypsin (3x crystallized) and Tween 20 (polyoxyethylene sorbitan monolaurate). Pronase (B grade) was purchased from California Corn. for Biochemical Research, Los Angeles, California; Rhozyme B-6 from Rohm and Haas Co., Philadelphia, Pennsylvania; hydroxylamine hydrochloride from Fisher Scientific Company, Fair Lawn, New Jersey; soybean trypsin inhibitor and density gradient grade sucrose from Mann Research Laboratories, New York, New York; and Genetron 113 from Allied
Chemical Co., Amherstburg, Ontario. Aspergillus-0 was made available by Dr. A. L. Tosoni, Connaught Medical Research Laboratories, Toronto, Ontario. Kethoxal (2-keto-3-ethoxy butyraldehyde hydrate) was kindly provided by Dr. L. E. Rhuland, The Upjohn Company, Kalamazoo, Michigan. Calf antiserum against the Indiana serotype of VS virus was supplied by Dr. R. G. Willis, Animal Diseases Research Institute, Hull, Quebec.

**Purification of Virus**

In a typical experiment, virus was purified in essentially three steps. First, the virus was pelleted from previously clarified infectious tissue culture fluid by centrifugation for 2 hours at 70,000 x g (Beckman Model L2-65B; Rotor no. SW 41 Ti). The pellets were resuspended in a small volume of Dulbecco's phosphate-buffered saline (PBS) minus magnesium and calcium salts, pH 7.4 (Merchant et al., 1964), and suspensions were combined. To further purify the virus
suspension, it was subjected to one or two treatments with fluorocarbon.

Such treatment consisted of shaking equal volumes of virus suspension
and Genetron [11] for approximately 1 minute, centrifuging for 5 minutes
at 3000 x g (Sorvall RC - 2B; Rotor no. SS - 34) and removing the
aqueous phase. This phase was carefully layered onto a preformed
discontinuous type of sucrose gradient (see appendix B) for the final
purification step. The gradient tubes were then centrifuged for 1 hour
at 85,000 x g (Beckman model L2-65B; Rotor no. SW 41 Ti). Infectivity
was associated with the strongly opalescent band, which appeared about
the middle of the tube. The opalescent band was removed by means of
a syringe fitted with a 16 gauge hypodermic needle. The purified virus
preparations had protein levels of about 25 ug/ml when assayed by the
method of Lowry et al., 1951).

Experimental Designs

(a) VS virus growth studies

For those experiments designed to study VS virus growth in the
presence of Kethoxal, 2-day old confluent cultures of either MDBK cells or L cells in 1 oz Brockway bottles were infected with VS virus at a multiplicity of 0.8. After adsorption for 45 minutes at 37°C, the cell layers were washed with Hank's balanced salt solution (HESS) (Merchant et al., 1964) to remove residual virus. The VS infected cultures were overlaid with MEM (control cultures) or MEM containing various concentrations of the antiviral and reincubated at 37°C. The experiments were terminated at 24 hours postinfection and stored at -60°C until assayed for infectivity.

(b) In vitro virucidal studies

For the study of the in vitro virucidal activity of Kethoxal, VS virus was initially purified as described in Materials and Methods, and then was exposed to chemical or enzymic treatment. Appropriate aliquots of purified virus and test compounds were mixed and incubated for 15 minutes at 37°C. All enzymes were solubilized in 0.05 M tris
(hydroxymethyl) aminomethane (Tris) buffer, pH 8.0, except trypsin and phospholipase preparations contained $10^{-3} \text{M CaCl}_2$. The chemical compounds, $\text{NH}_2\text{OH}, \text{HCl}$ and Tween 20, were dissolved in PBS, pH 7.4 and Tris buffer, pH 8.0, respectively. To remove the residual test compounds from the treated mixtures, the samples were centrifuged on a discontinuous sucrose gradient or extracted with fluorocarbon. Control preparations were treated in a similar fashion substituting buffer instead of the test compound. Equal volumes of treated and non-treated virus were then mixed with solutions of varying Ketoxal concentration, and incubated for 30 minutes at room temperature. Samples of the mixtures were assayed for infectivity. By using the infectivity values for enzyme-treated virus + Ketoxal (A), enzyme-treated virus (B), and untreated virus (C) the percent infectivity reduction can be calculated as follows:

\[
\frac{A - B}{C} \times 100 = \% \text{ infectivity reduction}
\]
Determination of Serum Neutralizing Activity

Serum neutralization tests were performed by dilution of the virus under test with 0.05 M Tris buffer, pH 8.0, to contain about 1200 PFU/ml. One ml of this virus was added to 1 ml of twofold serial dilutions of specific VS calf antiserum (previously inactivated for 30 minutes at 56°C) in Tris buffer. After incubation of the virus-antiserum mixtures for 1 hour at 37°C, they were chilled in ice until inoculated onto cell cultures for plaque assay. Three plates were used for each dilution of antiserum. Untreated control virus under the same conditions was assayed in parallel with the enzymatically - treated viral suspensions. The dilution of antiserum giving a 50% reduction in the number of plaques was taken as the end point.
EXPERIMENTAL RESULTS

Effect of Kethoxal on VS Virus Infectivity in Different Cell Lines

To evaluate the antiviral activity of Kethoxal against VS virus, replicate infected cultures of MDBK cells and L cells were overlaid with MEM containing varying concentrations of Kethoxal. After incubation for 24 hours at 37°C, infectious titers were determined.

As shown in Table 1, the viral yields from MDBK and L cells decreased as the concentration of Kethoxal was increased. Both cell lines overlaid with medium supplemented with a level of 50 μg/ml of Kethoxal produced viral yields of about 1% that obtained in control infected cultures. However, it appears that VS virus multiplication was more sensitive to Kethoxal in L cells than MDBK cells since the latter yielded about 66% more infectious virus in the presence of 5 μg/ml of the drug.
Table 1. The effect of varying concentrations of Kethoxal on infectious yields of vesicular stomatitis virus in different cell lines.*

<table>
<thead>
<tr>
<th>Concentration of Kethoxal (ug/ml)</th>
<th>Viral yields (PFU/ml)</th>
<th>Av. % inhib.</th>
<th>Viral yields (PFU/ml)</th>
<th>Av. % inhib.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDBK cells</td>
<td></td>
<td>L cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>0</td>
<td>9.0 x 10⁶</td>
<td>9.2 x 10⁶</td>
<td>6.4 x 10⁸</td>
<td>3.8 x 10⁸</td>
</tr>
<tr>
<td>5</td>
<td>8.0 x 10⁶</td>
<td>4.9 x 10⁶</td>
<td>5.8 x 10⁷</td>
<td>3.9 x 10⁷</td>
</tr>
<tr>
<td>25</td>
<td>2.1 x 10⁶</td>
<td>2.7 x 10⁶</td>
<td>5.2 x 10⁷</td>
<td>1.4 x 10⁷</td>
</tr>
<tr>
<td>50</td>
<td>1.9 x 10⁵</td>
<td>3.6 x 10⁴</td>
<td>2.6 x 10⁴</td>
<td>1.7 x 10⁴</td>
</tr>
<tr>
<td>75</td>
<td>N.D.</td>
<td>8.6 x 10³</td>
<td>1.8 x 10²</td>
<td>&lt;1.0 x 10²</td>
</tr>
<tr>
<td>100</td>
<td>1.1 x 10²</td>
<td>4.3 x 10²</td>
<td>&lt;1.0 x 10²</td>
<td>&lt;1.0 x 10²</td>
</tr>
</tbody>
</table>

* Cultures of MDBK and L cells were incubated with VS virus for 45 minutes, washed and exposed to different concentrations of Kethoxal in medium MEM. Control infected cultures were incubated in MEM. At 24 hours postinfection at 37°C, infectious viral yields were determined.
Effect of Kethoxal on VS Virus Infectivity in Different Growth Media

To determine whether different growth media influence the antiviral activity of Kethoxal against VS virus, MDEK cultures were overlaid with HBSS solution for 6 hours prior to virus inoculation to somewhat deplete residual intracellular metabolites. After virus adsorption, cultures were washed and divided into two sets. Duplicate cultures of one set were incubated with different levels of Kethoxal in medium MEM. The other set of cultures was similarly treated, but with MEM lacking cystine and methionine. Viral yields were determined following 24 hours incubation at 37°C.

Table 2 indicates that the omission of cystine and methionine from the growth medium effectively reduced the final yield of infectious virus about 80%. But when Kethoxal was incorporated in medium MEM lacking cystine and methionine, the inhibitory capacity of the drug at various concentration levels approached that observed in the presence of medium MEM.
Table 2. The effect of varying concentrations of Ketoxal on infectious yields of vesicular stomatitis virus in different growth media.

<table>
<thead>
<tr>
<th>Concentration of Ketoxal (ug/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>0</td>
<td>9.1 x 10⁶</td>
<td>-</td>
<td>1.9 x 10⁶</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>2.4 x 10⁶</td>
<td>73.4</td>
<td>2.3 x 10⁵</td>
<td>87.9</td>
</tr>
<tr>
<td>50</td>
<td>1.1 x 10⁵</td>
<td>93.8</td>
<td>7.3 x 10³</td>
<td>99.6</td>
</tr>
<tr>
<td>75</td>
<td>3.6 x 10³</td>
<td>99.9</td>
<td>1.2 x 10²</td>
<td>99.9</td>
</tr>
<tr>
<td>100</td>
<td>2.7 x 10²</td>
<td>99.9</td>
<td>2.6 x 10¹</td>
<td>99.9</td>
</tr>
</tbody>
</table>

*Six-hour starved cultures of MDCK cells were incubated with VS virus for 45 minutes, washed and overlaid with medium MEM or MEM lacking cystine and methionine. Both formulations of growth medium contained varying concentrations of Ketoxal. After incubation for 24 hours at 37°C, infectious viral yields were determined.*
Preparation of Purified Vesicular Stomatitis Virus

To exclude possible cellular contamination of virions for studies concerning surface structures, it was necessary to develop a procedure for the concentration and purification of VS virus. The procedure used is described in detail in Materials and Methods.

The results of this experimental procedure are shown in Table 3. Based on protein content, high speed centrifugation resulted in a 20 to 22-fold purification, while each fluorocarbon treatment increased the purification rate 9-fold. The viral preparation was purified an additional 600 to 800 times by passage through a discontinuous sucrose gradient.

Comparison of the protein content of VS virus before and after purification with the corresponding biological activity indicated that the virus infectivity/protein ratio was improved about 13 times over that of the initial infectious tissue culture fluid.

All further studies were carried out using virus prepared in the above manner.
Table 3. Purification of Vesicular Stomatitis Virus \(^a\)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Protein (μg/ml)</th>
<th>Purification rate</th>
<th>Virus infectivity (PFU/ml x 10^8)</th>
<th>Virus infectivity/protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clariified infectious fluid (44.0 ml)</td>
<td>18136</td>
<td>18136</td>
<td>8.6</td>
<td>8.6</td>
</tr>
<tr>
<td>70,000 x g pellet</td>
<td>833</td>
<td>900</td>
<td>22x</td>
<td>20x</td>
</tr>
<tr>
<td>Resuspended 70,000 x g pellet fluorocarbon treated 1x</td>
<td>575</td>
<td>625</td>
<td>31x</td>
<td>29x</td>
</tr>
<tr>
<td>Resuspended 70,000 x g pellet fluorocarbon treated 2x</td>
<td>450</td>
<td>490</td>
<td>40x</td>
<td>37x</td>
</tr>
<tr>
<td>Sucrose gradient purified virus</td>
<td>28</td>
<td>20</td>
<td>640x</td>
<td>900x</td>
</tr>
</tbody>
</table>

\(^a\) The purification procedure is described in detail under Materials and Methods.
Virucidal Activity of Ketoxal against VS Virus

In order to determine to what extent purified VS virus is sensitive to in vitro Ketoxal treatment, purified virus preparations were mixed with varying concentrations of the drug, as described in Materials and Methods, and assayed for infectious virus.

As illustrated in Figure 1, the virus preparation became progressively more susceptible to inactivation as the level of Ketoxal was increased. In addition, the data indicated that during the 30 minute test period virus infectivity dropped by 53% following exposure to only 5 μg/ml of the drug, while 99% reduction is achieved at a level of 75 μg/ml.

Virucidal Activity of Ketoxal against VS Virus following Chemical Pretreatment

Since the preceding experiment indicated that Ketoxal had a direct virucidal action on VS virus, it was of interest to examine whether the reactivity of the viral surface to Ketoxal could be affected by
Fig. 1. Virucidal activity of Ketoxal against VS virus. Samples of VS virus were mixed with varying concentrations of Ketoxal as described in Materials and Methods, and assayed for infectious virus.
by pretreatment with other chemical agents.

Equal volumes of purified virus and either 1% Tween 20 or 0.004 M \( \text{NH}_2\text{OH.HCl} \) were mixed and incubated for 15 minutes at 37°C. The virus-chemical mixtures were centrifuged in discontinuous gradients to reband the virus before exposure to Kethoxal and then titrated for infectious virus.

The results are shown in Figure 2. At the concentration levels tested, pretreatment of virus with Tween 20 or \( \text{NH}_2\text{OH.HCl} \) considerably depressed viral infectivity when compared with untreated controls.

When pretreated cultures were exposed to Kethoxal, the degree of virucidal activity was similar to that observed in Kethoxal-treated cultures (99%).
Fig. 2. Virucidal activity of Ketoxal against VS virus following chemical treatment. Virus preparations were treated with either 0.5% Tween 20 or 0.002 M NH₄OH·HCl, centrifuged on discontinuous sucrose gradients, exposed to 75 ug/ml of Ketoxal, and titrated for infectious virus. Solid bars indicate the infectivity of control preparations in each case; cross-hatched bars designate the presence of Ketoxal.
Sensitivity of Trypsin-treated VS Virus to Ketoxal

Because treatment with select chemical agents did not affect the reactivity of VS virus to Ketoxal, it was decided to attempt an enzymatic alteration of the surface structure of VS virus and in this way possibly modify the virucidal action of the drug.

To evaluate the sensitivity of trypsintreated VS virus to Ketoxal, virus preparations were initially treated with trypsin at levels of 0, 10, 50 and 100 ug/ml as described in Materials and Methods. All samples were then exposed to 75 ug/ml of Ketoxal and titrated for infectious virus.

From Table 1, it is clear that the infectivity of non-enzyme treated samples exposed to Ketoxal was reduced by 95%. However, virus pretreated with 50 or 100 ug/ml of trypsin appeared to be less sensitive to Ketoxal since the extent of infectivity reduction was only about 50%.

In view of these results, a comparison was made of VS virus infectivities obtained at different concentrations of trypsin and
Table 4. Sensitivity of VS virus to Ketoxal following trypsin treatment

<table>
<thead>
<tr>
<th>Concentration of enzyme (ug/ml)</th>
<th>Infectivity (PFU/ml)</th>
<th>Non-treated</th>
<th>Ketoxal-treated</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.5 x 10^6</td>
<td>3.0 x 10^5</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.7 x 10^6</td>
<td>1.4 x 10^5</td>
<td>97.9</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3.8 x 10^6</td>
<td>3.7 x 10^5</td>
<td>52.8</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.6 x 10^6</td>
<td>3.3 x 10^5</td>
<td>51.3</td>
<td></td>
</tr>
</tbody>
</table>

a Ketoxal was used at a final concentration of 75 ug/ml.
Kethoxal. Virus preparations were pretreated with trypsin as before, then exposed to Kethoxal at concentrations of 75, 37.5, 12.5 and 2.5 ug/ml and assayed for infectivity.

Fig. 3 indicates that, at each level tested, the virucidal activity of Kethoxal against trypsin-treated VS virus was reduced. Moreover, it appears that treatment of virus with trypsin levels higher than 50 ug/ml was ineffective in further modifying the virucidal activity of the drug.

Since it was demonstrated that pretreatment of VS virus with trypsin restricted the virucidal activity of Kethoxal, it suggested that some modification of the viral envelope or other exposed surfaces had occurred. To answer this question, it was believed that the use of serum neutralization tests might reveal surface structural changes. Virus preparations were initially treated with 50 ug/ml of trypsin by mixing equal volumes of virus and enzyme solution. Control samples were mixed with buffer only. After incubation for 15 minutes at 37°C, the samples
Fig. 3. Comparison of VS virus infectivities obtained at different concentrations of trypsin and Kethoxal. Virus preparations, initially exposed to 0, 10, 50 and 100 μg/ml of trypsin, were treated with Kethoxal at levels of 2.5, 12.5, 37.5 and 75 μg/ml and assayed for infectious virus. The points in the curves represent the % infectivity reduction at the enzyme level indicated following treatment with Kethoxal at levels of (X) 2.5, (▲) 12.5, (■) 37.5 and (○) 75 μg/ml.
were mixed with specific VS virus calf antiserum as described in Materials and Methods and the 50% end point was determined in each case. Thus, any change in the neutralizability of trypsin-treated and control samples could be related to differences in the surface structure of the virus particles.

The results (Figure 4) show that the neutralizability of the virus particles after enzyme treatment differed from untreated controls. To achieve a 50% reduction of plaques, neutralization of untreated particles occurred at a 1:5000 dilution of antiserum, whereas trypsin-treated particles only required an antiserum dilution of 1:7000. Repeated experiments confirmed the above results and indirectly implied some surface changes in VS particles.
Fig. 4. Serum neutralization tests. Preparations of untreated VS virus and virus pretreated with 50 μg/ml of trypsin were mixed with 2-fold serial dilutions of specific VS virus calf antiserum as described in Materials and Methods. All samples were assayed for infectivity. The points in the curves represent the % plaque reduction at the antiserum dilution indicated for (●) non-enzyme treated virus and (○) trypsin-treated samples. The antiserum titer giving a 50% reduction in plaque numbers was determined in each case.
Virucidal Activity of Ketoxal against VS Virus following Pretreatment with Different Proteolytic Enzymes

To gain more information on the capability of different proteolytic enzymes to alter the virucidal activity of Ketoxal, pronase was added at levels of 0, 2, 10 and 50 μg/ml to virus preparations, exposed to 75 μg/ml of Ketoxal and assayed for infectivity. The results (Table 5) indicate that pretreatment with 50 μg/ml of pronase substantially decreased the sensitivity of VS virus to Ketoxal since 67% more infectious virus was found with the pretreated preparation.

Based on the data obtained with trypsin and pronase, it was decided to compare the effect of α-, β- and γ-chymotryptsin, fungal protease (from Streptomyces griseus), Rhozyme B-6 (from Bacillus spp.), and Aspergillin-O (from Aspergillus oryzae) at a final concentration of 50 μg/ml. Table 6 shows that all enzymes tested, except β-chymotrypsin, markedly reduced the virucidal action of Ketoxal on VS virus. However, pretreatment of virus with α- and γ-chymotryptsins
Table 5. Sensitivity of VS virus to Kethoxal following pronase treatment

<table>
<thead>
<tr>
<th>Concentration of enzyme (µg/ml)</th>
<th>Infectivity (PFU/ml)</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>Kethoxal-treated(^a)</td>
</tr>
<tr>
<td>0</td>
<td>1.8 x 10^6</td>
<td>9.1 x 10^4</td>
</tr>
<tr>
<td>2</td>
<td>2.8 x 10^6</td>
<td>1.0 x 10^5</td>
</tr>
<tr>
<td>10</td>
<td>1.2 x 10^6</td>
<td>2.1 x 10^4</td>
</tr>
<tr>
<td>50</td>
<td>5.6 x 10^5</td>
<td>5.2 x 10^4</td>
</tr>
</tbody>
</table>

\(^a\) Kethoxal was used at a final concentration of 75 µg/ml.
Table 6. Sensitivity of VS virus to Ketoxal following treatment with proteolytic enzymes.

<table>
<thead>
<tr>
<th>Enzyme Pretreatment</th>
<th>Infectivity (PFU/ml)</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>Ketoxal-treated</td>
</tr>
<tr>
<td>none</td>
<td>$3.2 \times 10^6$</td>
<td>$1.7 \times 10^5$</td>
</tr>
<tr>
<td>$\alpha$ - chymotrypsin</td>
<td>$1.5 \times 10^6$</td>
<td>$9.0 \times 10^4$</td>
</tr>
<tr>
<td>$\beta$ - chymotrypsin</td>
<td>$2.9 \times 10^6$</td>
<td>$1.3 \times 10^5$</td>
</tr>
<tr>
<td>$\gamma$ - chymotrypsin</td>
<td>$1.4 \times 10^6$</td>
<td>$6.5 \times 10^4$</td>
</tr>
<tr>
<td>fungal protease (from Streptomyces griseus)</td>
<td>$8.6 \times 10^5$</td>
<td>$6.5 \times 10^4$</td>
</tr>
<tr>
<td>bacterial protease (Rhizome B-5 from Bacillus sps.)</td>
<td>$7.4 \times 10^5$</td>
<td>$3.6 \times 10^3$</td>
</tr>
<tr>
<td>Aspergillus - 0 (from Aspergillus oryzae)</td>
<td>$7.4 \times 10^5$</td>
<td>$3.3 \times 10^4$</td>
</tr>
</tbody>
</table>

<sup>a</sup> All enzymes were used at a level of 50 ug/ml.

<sup>b</sup> Ketoxal was used at a final concentration of 75 ug/ml.
was not as effective as bacterial and fungal proteases in reducing the virucidal activity of the drug.

**Effect of Pretreatment with Phospholipase C or Ribonuclease A on the Virucidal Activity of Ketoxal against VS Virus**

Further studies were performed to evaluate the antiviral activity of Ketoxal against VS virus pretreated with either phospholipase C or ribonuclease A (RNase A).

Virus preparations were mixed with 2, 20 and 200 ug/ml of phospholipase C or with RNase A at levels of 10, 100 and 200 ug/ml, subjected to Ketoxal at a concentration of 75 ug/ml and assayed for infectious virus.

As shown in Table 7, pretreatment of VS virus with low levels (2 and 20 ug/ml) of phospholipase C decreased Ketoxal activity to about the same extent. However, the virucidal action of the drug was greatly reduced when the virus was pretreated with 200 ug/ml of the same enzyme. In contrast, the antiviral activity of Ketoxal against virus,
Table 7. Effect of pretreatment with phospholipase C or ribonuclease A on the virucidal activity of Ketoxal against VS virus

<table>
<thead>
<tr>
<th>Enzyme pretreatment</th>
<th>Non-treated</th>
<th>Ketoxal-treated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Infectivity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>95.1</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µg/ml</td>
<td>6.1 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.3 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>66.8</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>6.1 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.7 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>66.9</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>1.2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>13.2</td>
</tr>
<tr>
<td>RNase A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>1.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>95.1</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>1.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.6 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>89.0</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>1.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>7.3 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>92.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ketoxal was used at a final concentration of 75 µg/ml.
following exposure to RNase, was unchanged at each enzyme level tested.

**Inability of Trypsin to Restore Infectivity of Kethoxal-Treated VS Virus**

An attempt was made to restore the infectivity of VS virus, initially treated with Kethoxal, by subsequent trypsin treatment.

Purified virus was treated with Kethoxal as described in Materials and Methods, and preparations were centrifuged for 2 hours at 70,000 x g (Beckman model L2-65B; Rotor no. SW 27 Ti). The virus pellets obtained were resuspended in 0.05 M Tris, pH 8.0, and treated with 50 ug/ml of trypsin for 15 minutes at 37°C in the presence of 10^{-3} M CaCl₂. To neutralize residual trypsin, soybean inhibitor was then added at a final concentration of 100 ug/ml. Following incubation for an additional 2 minutes at room temperature, the virus preparations were assayed for infectivity.

The results (Table 8) indicate that the infectivity of VS virus, initially treated with Kethoxal, was not restored by subsequent trypsin treatment.
Table 8. Inability to restore infectivity of Ketoxal-treated VS virus by trypsin treatment *

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infectivity (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$3.2 \times 10^7$</td>
</tr>
<tr>
<td>Ketoxal (75 µg/ml)</td>
<td>$1.5 \times 10^4$</td>
</tr>
<tr>
<td>Ketoxal (75 µg/ml) + trypsin (50 µg/ml)</td>
<td>$9.6 \times 10^3$</td>
</tr>
</tbody>
</table>

* A purified VS virus suspension was divided into two fractions, and one of these was treated with Ketoxal as described in Materials and Methods. The preparations were centrifuged at 70,000 x g for 2 hours, and the appropriate samples were treated with trypsin (50 µg/ml) for 15 minutes at 37°C before the addition of trypsin soybean inhibitor (100 µg/ml) and then assayed for infectivity.
DISCUSSION

The antiviral activity of Ketoxal against vesicular stomatitis virus has been studied using two *in vitro* tests: (a) the growth cycle experiment in cell culture, which evaluates antiviral effects related to parameters of virus multiplication and (b) the virucidal test which measures the degree of direct inactivation (Grunberg & Prince, 1970).

The structure of Ketoxal is shown below:

\[
\begin{array}{c}
\text{O} & \text{O} \\
\text{CH}_3 - \text{CH} - \text{C} - \text{CH} - \text{H}_2\text{O} \\
\hline
\text{OC}_2\text{H}_5 & \text{O} & \text{O} \\
\hline
\end{array}
\]

The terminal α-ketoaldehyde grouping, \(-\text{C} - \text{CH}_2\), is thought to be the reactive site of the molecule (McLimans *et al.*, 1957). Evidence presented here shows that when Ketoxal is incorporated in the virus growth medium, VS viral yields from MBRK and L cells progressively decrease with increasing amounts of drug. This agrees with the results of Renis (1970), who reported inhibition of Coxsackie A-21 virus replication in monolayers of ML cells overlaid with medium containing...
Kethoxal. To establish a clearer definition of drug effect relating to environmental influences on cells, it was found that the omission of the sulphur containing amino acids, cystine and methionine, from the virus growth fluid has little effect on the antiviral action of Kethoxal.

In experiments reported earlier, the in vitro virucidal activity of Kethoxal against a number of DNA and RNA viruses (Underwood & Weed, 1961; Renis, 1970) was demonstrated using serum-free tissue culture infectious fluids. Since the major interest was in studying the sensitivity of VS virus to Kethoxal, a purification procedure involving high speed centrifugation, fluorocarbon treatment and discontinuous sucrose gradient centrifugation was devised to exclude medium constituents and minimize cellular contamination of virions. This procedure routinely yielded virions which were purified about 750 times and still sensitive to inactivation in vitro by Kethoxal.

Among the proteolytic enzymes, trypsin has been used to obtain viral subunits (Simpson & Hauser, 1966; Maeno et al., 1970), to enhance virus infectivity (Spendlove & Schaffer, 1965; Spendlove et al., 1970).
and enzymic activity (Aubertin & McAuslan, 1972) and to alter viral surface morphology (Biddle, 1963; Kendall et al., 1969). Because of these reports and the findings of Cartwright et al. (1969) and McCombs et al. (1966), who have shown that trypsin removes the external spike-like projections of VS virus, trypsin was studied more closely with regard to its effect on the surface of VS particles. Using serum neutralization tests, trypsin-treated VS virions required less virus-specific antiserum than did control samples to achieve a 50% reduction in plaques. This finding provides indirect evidence that the viral surface is modified. Furthermore, pretreatment of VS virus with trypsin reduced the sensitivity of the virions to the virucidal activity of Kethoxal.

Since trypsin catalyzes the hydrolysis of bonds involving arginine or lysine residues, a possible Kethoxal reaction site is suggested. In support of this, Underwood (1959) has observed the extremely rapid reaction of the drug with arginine as well as a slower binding to lysine. Attempts to restore the infectivity of VS virus, initially treated with Kethoxal, by subsequent trypsin treatment were unsuccessful. If the
virucidal activity of Kethoxal is a result of binding to the surface of the virus particle, the bond formed is apparently inaccessible or insensitive to trypsic action.

In contrast to trypsin, the non-specific proteolytic enzyme, pronase, will digest almost any protein to free amino acids (Nomoto et al., 1960). Mild pretreatment of VS virus with this enzyme substantially reduced the virucidal activity of Kethoxal. However, comparison of the data obtained with trypsin and pronase reveals that the extent of virucidal action of the drug after pretreatment with a non-specific enzyme was only slightly greater than that with trypsin.

Pretreatment of virus with α- and γ- chymotrypsins, Rhzyme B-6 and Aspergillin-0 prior to Kethoxal treatment gave results similar to those obtained with trypsin and pronase. Both α- and γ- chymotrypsins differ, however, from β- chymotrypsin in their effectiveness in reducing the virucidal action of the drug. Underwood (1959) has reported the binding of Kethoxal to the aromatic amino acid, tryptophane, which when present in peptide linkages is a major site of
chymotrypsin action. This suggests another site of Kethoxal action. The fact that sensitivity of VS virions to the action of Kethoxal could be reduced by pretreatment with proteolytic enzymes has provided suggestive evidence that binding sites are available on the envelope or substructure of the virion.

Phospholipase C, which catalyzes the hydrolysis of the linkage between glycerol and phosphate, has been used to study the structure of a number of viruses (Mizutani & Mizutani, 1964; Simpson & Hauser, 1966). Pretreatment of VS virus with low levels of phospholipase C slightly reduced the virucidal activity of Kethoxal. However, a high phospholipase concentration caused a marked decrease in antiviral activity. Cartwright et al. (1969) have shown that the spike structure of the surface was unaffected by phospholipase C, but the remainder of the surface, presumably phospholipid components located in the regions between the spikes, was digested. Since the sensitivity of VS virus to Kethoxal was reduced by phospholipase pretreatment, the integrity of the surface phospholipid component may be a requirement for optimum
virucidal activity.

Since it has been reported that ribonuclease was without effect on surfaces of intact influenza (Mizutani & Mizutani, 1964) and VS Simpson & Hauser, 1966) particles, pretreatment of intact VS virions with RNase should disclose no surface structure modification nor a change in the effectiveness of Kethoxal treatment. Experiments have shown that RNase-treated VS virus is equally as sensitive to drug treatment as control preparations.

Previous chemical studies on the exposure of myxoviruses to the dispersal agent, Tween 20, resulted in a dissociation of the virions into biologically active subunits (Webster and Darlington, 1969). The present study demonstrates that mild Tween 20 treatment of VS virus considerably depressed viral infectivity, but did not affect the relative virucidal activity of Kethoxal. Apparently such treatment does not alter the surface groupings sensitive to drug activity.

Similar results were achieved with the chemical agent, hydroxylamine,
which has been shown to inactivate influenza virus by attacking the
pyrimidines of the viral nucleic acid (Scholitissek & Rott, 1963)
and certain bacteriophages by splitting the thiolester bonds in their
protein component (Kozloff et al., 1957).

The present study has shown that the antiviral activity of
Kethoxal against vesicular stomatitis virus can be modified to a
limited extent by pretreatment of virions with various enzymes
suggesting a possible drug mechanism. Further chemical studies may
reveal the exact mode of action by which Kethoxal exerts this virucidal
effect.
SUMMARY

The antiviral activity of Ketoxal against vesicular stomatitis virus has been studied. VS virus growth studies have shown virus multiplication in different cells and in different growth media was inhibited by the drug. Ketoxal exhibited potent virucidal activity against highly purified VS virus preparations. When proteolytic enzymes including fungal and bacterial proteases, \( \alpha \)- and \( \beta \)-chymotrypsins and trypsin were used to modify the viral surface structure, VS virus sensitivity to the antiviral activity of the drug was considerably reduced. In contrast, \( \beta \)-chymotrypsin-treated and untreated virus preparations were equally susceptible to Ketoxal treatment. The infectivity of Ketoxal-treated VS virus could not be regenerated by subsequent trypsin treatment. A slight depression of virucidal activity was achieved when VS virus was pretreated with phospholipase C.

Exposure of the virus to RNase prior to drug treatment had no effect on virucidal activity. Although virus infectivity was reduced by
pretreatment with the chemical agents, Tween 20 and NH₂OH·HCl, the relative antiviral action of Kethoxal was not affected.

The results indicate that Kethoxal possesses two modes of antiviral action: (1) inhibition of intracellular virus multiplication and
(2) direct virucidal action on extracellular virus.
APPENDIX A

Sterilization of Centrifuge Tubes

Sterilization of cellulose nitrate tubes (no. 331370, Spinco Division of Beckman Instruments, Inc., Palo Alto, California) was achieved by immersing the tubes for 10 minutes in a 0.2% sterile solution of benzylkonium chloride followed by exposure of the emptied tubes to ultraviolet light for 12 hours.
APPENDIX B

Preparation of Discontinuous Gradients

Discontinuous gradient columns were prepared by floating layers of 1.5, 1.5, 1.5, 3.0 and 3.0 ml of solutions of 50, 200, 300, 400 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 overnight at 4°C.
REFERENCES


VITA AUCTORIS

Born:


Daughter of Mr. & Mrs. G.G. Morrow

Elementary Education:

Ivor Chandler Public School

Windsor, Ontario. 1954 - 1961

Secondary Education:

Vincent Massey Collegiate Institute

Windsor, Ontario. 1961 - 1966

University Education:

University of Windsor


Graduated with the degree of Bachelor of Science in Biology