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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
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Ⓒ D. Gail Morrow 1972

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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 γ- 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 $NH_2OH \cdot HCl$, 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.

ii

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iii

TABLE OF CONTENTS

LIST OF TABLES

v

LIST OF ILLUSTRATIONS

vi

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 (Fenner & 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 β - thiosemicarbazone (Marboran); 5 - iodo - 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

1

compounds with a terminal α- ketoaldehyde or α- 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, 1968). 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 (Staehelin, 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 (Ranis, 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 mm. 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

5

cell. After adsorption at 37°C for 45 minutes, medium ELAY - 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 x 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 $37^{\circ}C$ in a

water saturated atmosphere of 5% CO_2 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 Cl. 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

Corp. 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. Aspergillin-O 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. N. 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 113 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 (HBSS) (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}M $CaCl_2$. The chemical

compounds, $NH_2OH.HCl$ 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 Kethoxal 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 + Kethoxal (A), enzyme-treated virus (B), and

untreated virus (C) the per·cent infectivity reduction can be

calculated as follows:

$$\frac{A - B}{C} \times 100 = \% \text{ infectivity reduction}$$

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 ug/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 ug/ml
of the drug.

13

Table 1. The effect of varying concentrations of Kethoxal on infectious yields of vesicular stomatitis virus in different cell lines.*

Concentration of Kethoxal (ug/ml)	MDBK cells Viral yields (PFU/ml)			L cells Viral yields (PFU/ml)		
	Trial 1	Trial 2	Av. % inhib.	Trial 1	Trial 2	Av. % inhib.
0	9.0×10^6	9.2×10^6	-	6.4×10^8	3.8×10^8	-
5	8.0×10^6	4.9×10^6	24	5.8×10^7	3.9×10^7	90
25	2.1×10^6	2.7×10^6	74	5.2×10^7	1.4×10^7	94
50	1.9×10^5	3.6×10^4	98	2.6×10^4	1.7×10^4	99
75	N.D.	8.6×10^3	99	1.8×10^2	$<1.0 \times 10^2$	99
100	1.1×10^2	4.3×10^2	99	$<1.0 \times 10^2$	$<1.0 \times 10^2$	99

* 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, MDBK cultures were overlaid with solution HBSS 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 $37^\circ C$.

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 Kethoxal on infectious yields of vesicular stomatitis virus in different growth media*

Concentration of Kethoxal (ug/ml)	MEM (complete)		MEM (minus cystine & methionine)	
	Infectivity (PFU/ml)	% Infectivity reduction	Infectivity (PFU/ml)	% Infectivity reduction
0	0.1×10^6	-	1.9×10^6	-
25	2.4×10^6	73.4	2.3×10^5	87.9
50	1.1×10^5	98.8	7.3×10^3	99.6
75	8.6×10^3	99.9	1.2×10^2	99.9
100	2.7×10^2	99.9	2.6×10^1	99.9

*Six-hour starved cultures of MDBK 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 Kethoxal. 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 43 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]

	Protein (ug/ml)		Purification rate		Virus infectivity (PFU/ml x 10^8)		Virus infectivity/ protein ratio	
Trial	1	2	1	2	1	2	1	2
Clarified infectious fluid (44.0 ml)	18136	18136	-	-	8.6	8.6	1	2
70,000 x g pellet	833	900	22x	20x	5.3	8.1	13	19
Resuspended 70,000 x g pellet fluorocarbon treated 1x	575	625	31x	29x	3.7	3.7	14	13
Resuspended 70,000 x g pellet fluorocarbon treated 2x	450	490	40x	37x	2.9	2.4	14	10
Sucrose gradient purified virus	28	20	640x	900x	0.6	0.4	45	42

[a] The purification procedure is described in detail under Materials and Methods.

Virucidal Activity of Kethoxal against VS Virus

In order to determine to what extent purified VS virus is sensitive to in vitro Kethoxal 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 Kethoxal was increased. In addition, the data indicated that during the 30 minute test period virus infectivity dropped by 53% following exposure to only 5 ug/ml of the drug, while 99% reduction is achieved at a level of 75 ug/ml.

Virucidal Activity of Kethoxal against VS Virus following Chemical Pretreatment

Since the preceding experiment indicated that Kethoxal had a direct virucidal action on VS virus, it was of interest to examine whether the reactivity of the viral surface to Kethoxal could be affected by

Fig. 1. Virucidal activity of Kethoxal against VS virus. Samples

of VS virus were mixed with varying concentrations of Kethoxal as

described in Materials and Methods, and assayed for infectious virus.
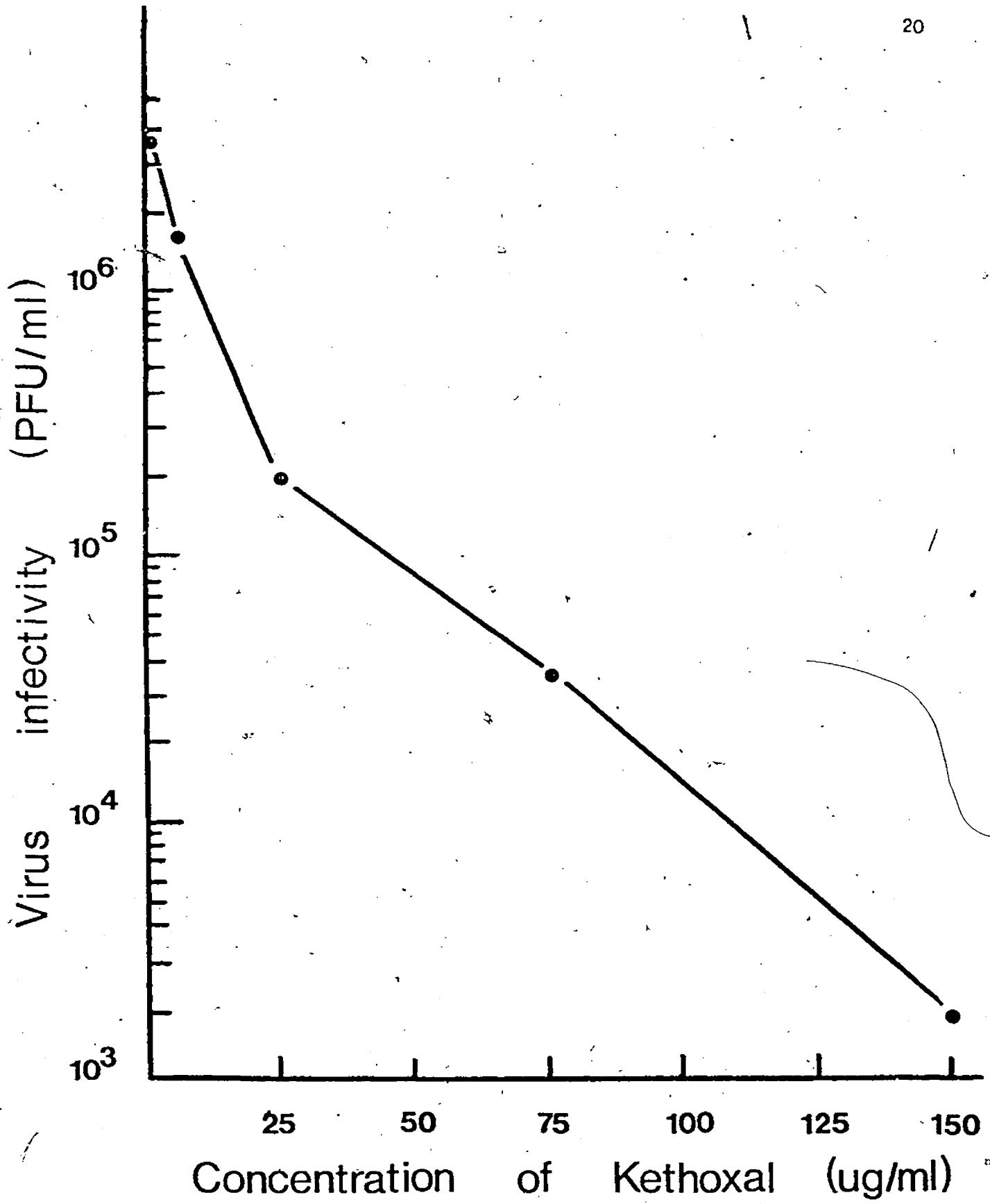
by pretreatment with other chemical agents.

Equal volumes of purified virus and either 1% Tween 20 or 0.004M NH$_2$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 NH$_2$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 Kethoxal against VS virus following

chemical treatment. Virus preparations were treated with either

0.5% Tween 20 or 0.002 M $NH_2OH.HCl$, centrifuged on discontinuous sucrose

gradients, exposed to 75 ug/ml of Kethoxal, and titrated for infectious

virus. Solid bars indicate the infectivity of control preparations in

each case; cross-hatched bars designate the presence of Kethoxal.

none   Tween20   NH$_2$OH

Pretreatment

Virus infectivity (PFU/ml)

10^7 10^6 10^5 10^4 10^3 10^2
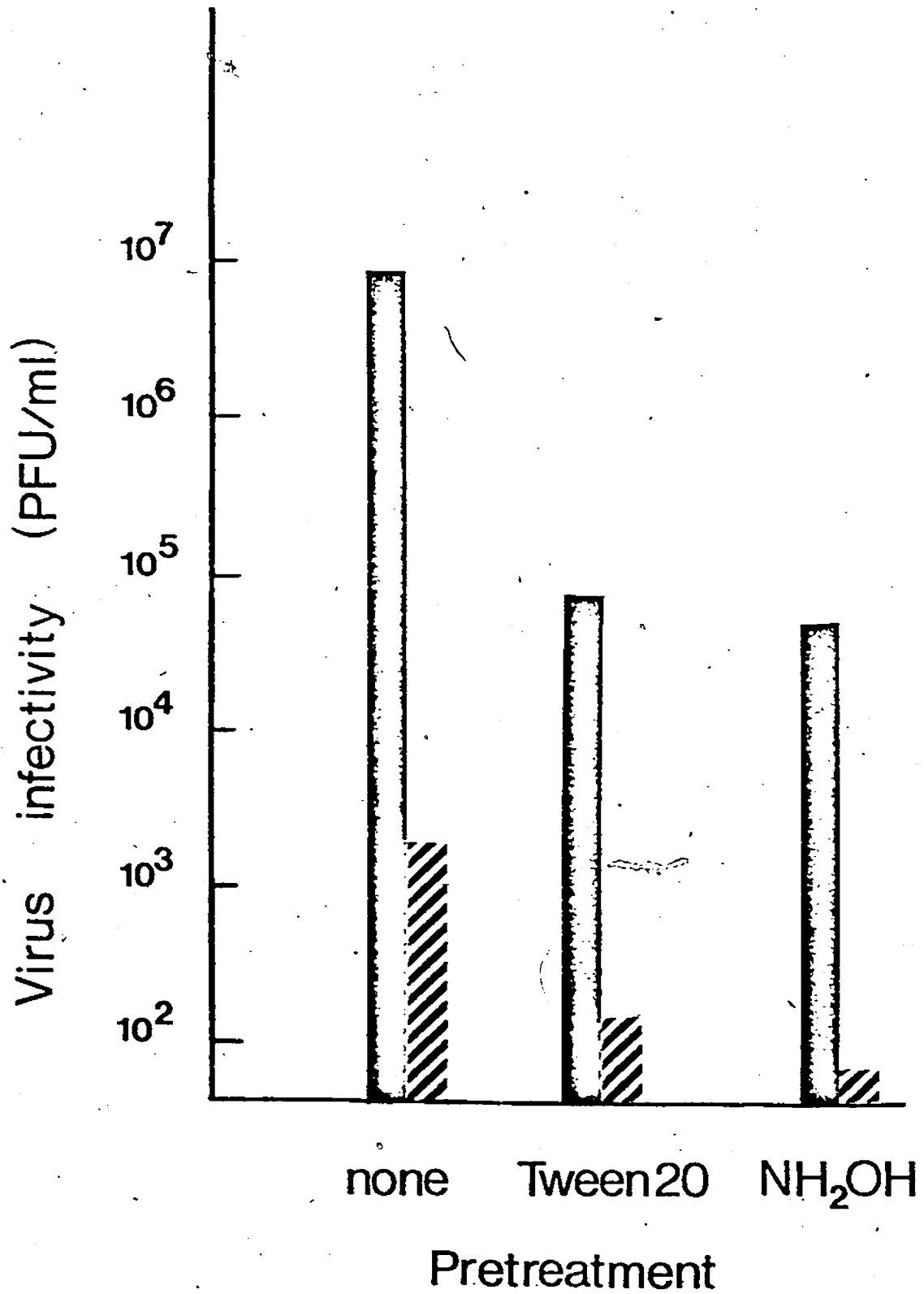
Sensitivity of Trypsin-treated VS Virus to Kethoxal

Because treatment with select chemical agents did not affect the reactivity of VS virus to Kethoxal, 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 trypsin-treated VS virus to Kethoxal, 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 Kethoxal and titrated for infectious virus.

From Table 4 it is clear that the infectivity of non-enzyme treated samples exposed to Kethoxal was reduced by 95%. However, virus pretreated with 50 or 100 ug/ml of trypsin appeared to be less sensitive to Kethoxal 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 Kethoxal following trypsin treatment

| Concentration of enzyme (ug/ml)	Infectivity (PFU/ml)		% Infectivity reduction
	Non-treated	Kethoxal-treated[a]	
0	6.5×10^6	3.0×10^5	95.0
10	6.7×10^6	1.4×10^5	97.9
50	3.8×10^6	3.7×10^5	52.8
100	3.6×10^6	3.3×10^5	51.3

[a] Kethoxal 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 ug/ml of trypsin, were treated with Kethoxal

at levels of 2.5, 12.5, 37.5 and 75 ug/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 ug/ml.
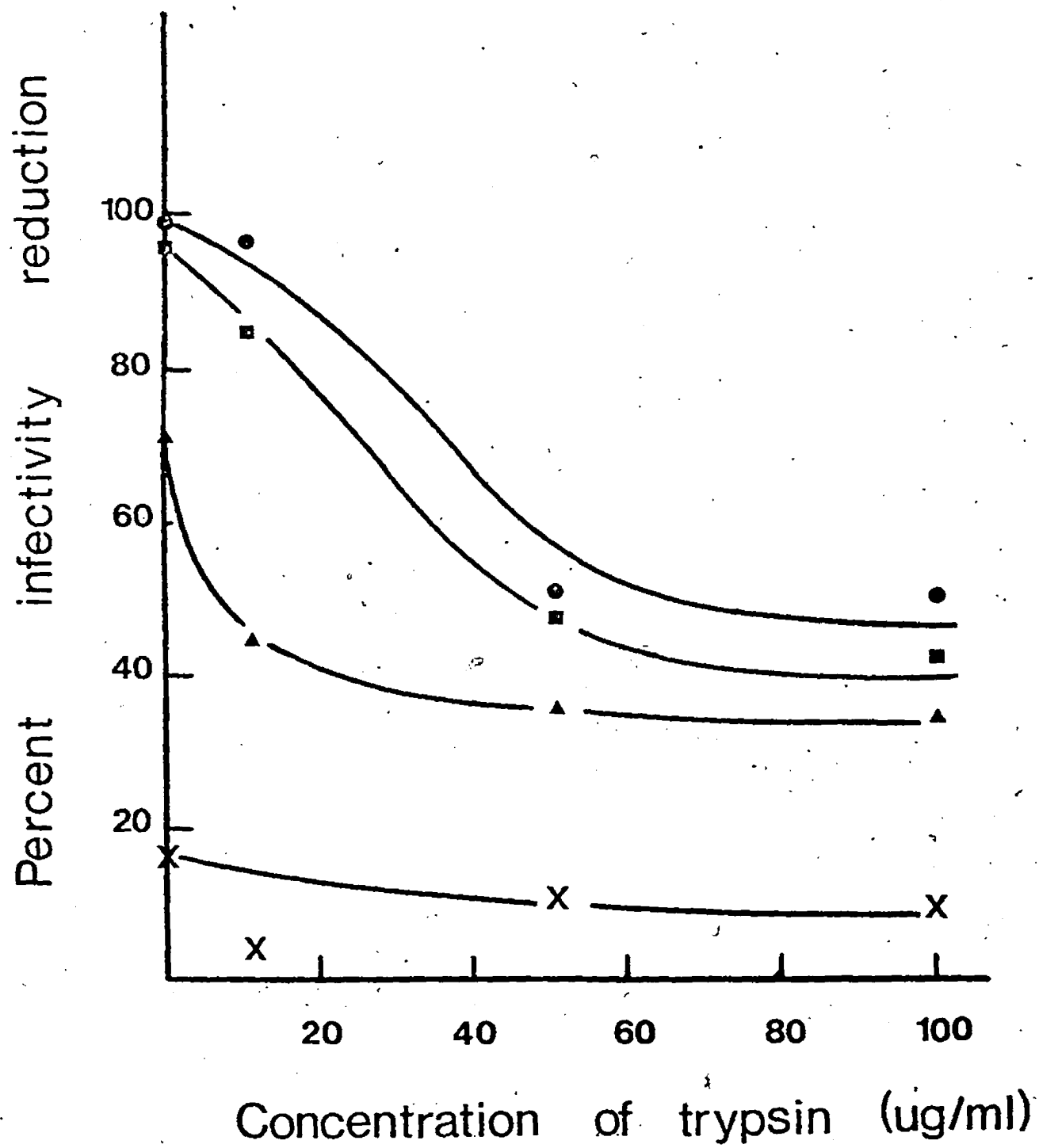
were mixed with specific VS virus calf antiserum as described in Materials

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 ug/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

(O) trypsin-treated samples. The antiserum titer giving a 50%

reduction in plaque numbers was determined in each case.
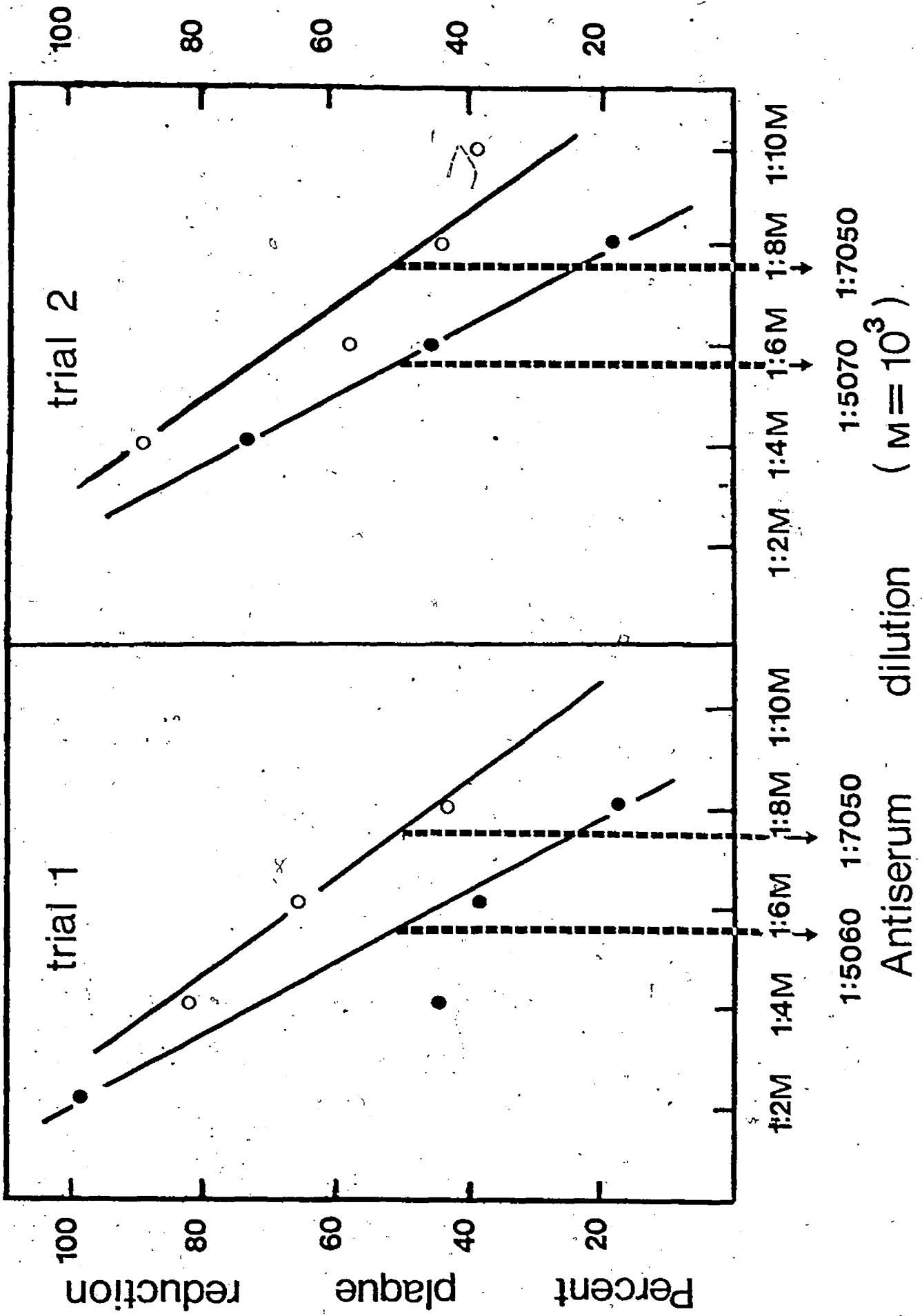
Virucidal Activity of Kethoxal against VS Virus following Pretreatment with Different Proteolytic Enzymes

To gain more information on the capability of different proteolytic enzymes to alter the virucidal activity of Kethoxal, pronase was added at levels of 0, 2, 10 and 50 ug/ml to virus preparations, exposed to 75 ug/ml of Kethoxal and assayed for infectivity. The results (Table 5) indicate that pretreatment with 50 ug/ml of pronase substantially decreased the sensitivity of VS virus to Kethoxal 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- chymotrypsins, fungal protease (from Streptomyces griseus), Rhozyme B-6 (from Bacillus sps.), and Aspergillin-O (from Aspergillus oryzae) at a final concentration of 50 ug/ml. Table 6 shows that all enzymes tested, except β- chymotrypsin, markedly reduced the virucidal action of Kethoxal on VS virus. However, pretreatment of virus with α- and γ- chymotrypsins

Table 5. Sensitivity of VS virus to Kethoxal following pronase treatment

Concentration of enzyme (ug/ml)	Infectivity (PFU/ml)		% Infectivity reduction
	Non-treated	Kethoxal-treated[a]	
0	1.8×10^6	9.1×10^4	94.9
2	2.8×10^6	1.0×10^5	94.4
10	1.2×10^6	2.1×10^4	95.5
50	5.6×10^5	5.2×10^4	28.2

[a] Kethoxal was used at a final concentration of 75 ug/ml.

Table 6. Sensitivity of VS virus to Kethoxal following treatment with proteolytic enzymes.

	Infectivity (PFU/ml)		
Enzyme Pretreatment[a]	Non-treated	Kethoxal-treated[b]	% Infectivity reduction
none	3.2×10^6	1.7×10^5	94.7
α - chymotrypsin	1.5×10^6	9.0×10^4	44.1
β - chymotrypsin	2.9×10^6	1.3×10^5	86.5
γ - chymotrypsin	1.4×10^6	6.5×10^4	41.9
fungal protease (from Streptomyces griseus)	8.6×10^5	6.5×10^4	24.8
bacterial protease (Rhozyme B-6 from Bacillus sps.)	7.4×10^5	3.6×10^3	23.0
Aspergillin - O (from Aspergillus oryzae)	7.4×10^5	3.3×10^4	22.0

[a] All enzymes were used at a level of 50 ug/ml.

[b] Kethoxal 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 Kethoxal against VS Virus

Further studies were performed to evaluate the antiviral activity of Kethoxal 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 Kethoxal 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 Kethoxal 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 Kethoxal against virus,

Table 7. Effect of pretreatment with phospholipase C or ribonuclease A on the virucidal activity of Kethoxal against VS virus

	Infectivity (PFU/ml)		
Enzyme pretreatment	Non-treated	Kethoxal-treated[a]	% Infectivity reduction
None	9.0×10^5	4.4×10^4	95.1
Phospholipase C			
2 ug/ml	6.1×10^5	8.3×10^3	66.8
20 ug/ml	6.1×10^5	7.7×10^3	66.9
200 ug/ml	1.2×10^5	1.0×10^2	13.2
RNase A			
10 ug/ml	1.9×10^6	4.5×10^4	95.1
100 ug/ml	1.0×10^6	8.6×10^3	89.0
200 ug/ml	1.9×10^6	7.3×10^4	92.0

[a] Kethoxal was used at a final concentration of 75 ug/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 41 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 $37^{\circ}C$ in the presence of 10^{-3} M $CaCl_2$. 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 Kethoxal-treated VS virus
by tryosin treatment *

Treatment	Infectivity (PFU/ml)
None	3.2×10^7
Kethoxal (75 ug/ml)	1.4×10^4
Kethoxal (75 ug/ml) + trypsin (50 ug/ml)	9.6×10^3

* A purified VS virus suspension was divided into two fractions, and one
of these was treated with Kethoxal 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 ug/ml) for 15 minutes
at $37^{\circ}C$ before the addition of trypsin soybean inhibitor (100 ug/ml)
and then assayed for infectivity.

DISCUSSION

The antiviral activity of Kethoxal 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 Kethoxal is shown below:

$$CH_3 - CH - \overset{\overset{\displaystyle O}{\|}}{C} - \overset{\overset{\displaystyle O}{\|}}{CH} \cdot H_2O$$
$$\underset{OC_2H_5}{|}$$
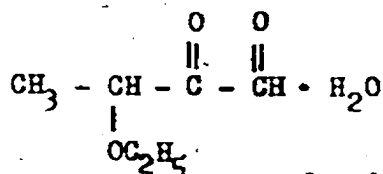
The terminal α- ketoaldehyde grouping, $- \overset{\overset{\displaystyle O}{\|}}{C} - \overset{\overset{\displaystyle O}{\|}}{CH}$, is thought to be

the reactive site of the molecule (McLimans et al., 1957). Evidence

presented here shows that when Kethoxal is incorporated in the virus

growth medium, VS viral yields from MDBK 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

36

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, 1968; Kendal 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 tryptic 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, Rhozyme 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 Kethoxal 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. Kethoxal exhibited potent virucidal activity against highly purified VS virus preparations. When proteolytic enzymes including fungal and bacterial proteases, α- and γ- 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, β- chymotrypsin-treated and untreated virus preparations were equally susceptible to Kethoxal treatment. The infectivity of Kethoxal-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

43

pretreatment with the chemical agents, Tween 20 and $NH_2OH \cdot HCl$, 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.

45

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 $4^{\circ}C$.

46

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47

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52