THE INACTIVATION OF HERPES SIMPLEX VIRUS BY PHOSPHATASE ENZYMES*

By HAROLD AMOS,‡ Ph.D.

(From the Department of Bacteriology and Immunology, Harvard Medical School, Boston)

(Received for publication, April 20, 1953)

In the course of the study of factors influencing the "spontaneous" inactivation of the virus of herpes simplex, it was found that phosphatase enzymes of the propagating tissue play a significant role in this inactivation and that the rate of deterioration can be markedly increased by addition of purified phosphatases to suspensions of this virus.

Methods and Materials

Preparation of virus.—The virus used was the LF strain of herpes simplex which had been maintained by mouse brain passage for a number of years. The virus is highly infectious for mice and produces characteristic pocks on the chorio-allantoic membrane of the chick embryo. Pools were prepared by grinding refrigerated, freshly removed brains with alundum in a mortar, suspending the mash in four times its weight of distilled water, physiological saline, or 0.03M phosphate buffer at pH 7.0–7.2 to give a 20 per cent suspension. The supernatant from centrifugation at 2000 R.P.M. for 20 minutes was frozen rapidly and stored as stock virus in sealed pyrex tubes in a dry ice refrigerator. Material to be used for experimental purposes was thawed, diluted with an equal volume of diluent, and filtered through a medium grade sintered glass filter.

Virus suspensions after filtration usually contained from 10^4 to $10^{4.5}$ LD_{50} per 0.05 ml. for mice inoculated intracerebrally. The number of infective units estimated by titration on the chorio-allantoic membrane of the chick embryo agrees rather well with the estimation by titration with mice.

Virus propagated on the chorio-allantoic membrane was prepared in the same manner as mouse brain suspensions. The quantity of virus by infective titer per unit weight of tissue was of the same order for both sources. Allantoic and amniotic fluid virus was harvested 48 hours after inoculation of 8 to 10 day embryos by the amniotic route (1).

Quantitative Estimation of Viral Infectivity.—Estimation of infectivity by counting plaques formed on the chorio-allantoic membrane was adopted as the standard procedure for this study, though periodic "reference" titrations were made by intracerebral inoculation of mice. The technique for plaque estimation was essentially that of Burnet and Beveridge (1) confirmed for herpes simplex by Shaffer and Enders (2).

Phosphate Determination.—Inorganic phosphate was determined by the method of Fiske and Subbarow (3).

* The studies reported in this paper were taken from a thesis submitted by the author to the Faculty of the Graduate School of Arts and Sciences of the Harvard University, Division of Medical Sciences, in partial fulfillment of the degree of Dóctor of Philosophy.

‡ Predoctorate Research Fellow, National Institutes of Health, Public Health Service, 1950–51.

Measurement of Phosphatase Activity.—Measurement of the phosphatase activity of normal tissue and of virus suspensions was carried out as follows: 1 ml. samples of cell-free suspensions from tissue or of allantoic fluid were added to 3.0 ml. of buffer (phthalate buffer pH 4.6 or borate buffer pH 8.6) containing an excess of Mg^{++} and of substrate (adenosine-3-phosphate or β-glycerophosphate). Samples were made up to 5.0 ml. with saline and incubated in a water bath at 37°C. for 4 hours. The phosphatase action was stopped at the end of the incubation with 10 per cent trichloracetic acid and inorganic phosphate estimated with appropriate blanks.

Purified Acid and Alkaline Phosphatase.—Purified acid (prostate) and alkaline (intestinal) phosphatases were obtained through the generosity of Dr. Gerhard Schmidt of Tufts Medical School (4, 10). These were highly active preparations, 0.01 ml. of which would hydrolyze 0.3 mg. to 0.7 mg. of phosphate from 50 mg. of adenosine-3-phosphate in 15 minutes at 37°C. in the presence of an excess of Mg^{++}.

The preparations of alkaline phosphatase were contaminated with traces of adenosine-deaminase (10) and would hydrolyze the following substrates (4, 10): α- and β-glycerophosphate;[1] yeast and muscle adenylic acid;[1] glucose-1-phosphate; glucose-6-phosphate; fructose 1,6-diphosphate; fructose-6-phosphate; RNA;[1] inositol monophosphate;[1] phosphopyruvic acid; pyrophosphoric acid; adenylpyrophosphoric acid; diphenylphosphoric acid. Cephalin,[1] lecithin,[1] sphingomyelin, and DNA[1] were not attacked. Preparations could not be shown to possess esterase activity for methyl butyrate[1] or olive oil.[1]

The preparations of acid phosphatase were contaminated with traces of ribonuclease (10) and would hydrolyze the following substrates: α- and β-glycerophosphate;[1] yeast and muscle adenylic acid;[1] RNA[1] (very slowly); glucose-6-phosphate; glucose-1-phosphate; fructose-6-phosphate. DNA, fructose 1,6-diphosphate, cephalin,[1] lecithin[1] were not attacked. Preparations could not be shown to possess esterase activity for methyl butyrate[1] or olive oil.[1,2]

EXPERIMENTAL RESULTS

Normal Rate of Inactivation.—Virus suspensions from tissue or in allantoic fluid were found to lose their infectivity within a period of 25 to 30 hours at 37°C. The time required for total inactivation under these conditions is a function of temperature and of the initial concentration of virus.

Phosphatase Activity of Normal and Infected Tissue Suspensions.—Determination of both acid and alkaline phosphatase activity of normal and of infected tissue suspensions revealed some activity for all suspensions tested. Table I presents the values obtained for normal and infected tissue preparations and fluids for the substrates indicated. From these measurements there appears to be no significant difference between the phosphatase activity of normal and infected tissues and fluids.

Effect of Phosphatase Inhibitors and Substrates on Survival of Virus.—The addition of compounds known to inhibit phosphatases (certain α-amino acids (5) and inorganic phosphate) and of phosphatase substrates (β-glycerophosphate and adenosine-3-phosphate) resulted in increasing the survival time of virus compared to survival in the absence of these compounds (Table II).

[1] The activity of the enzyme preparations toward these substrates was tested by the author in the course of the present work.

[2] Dr. Gerhard Schmidt, Tufts Medical School, kindly consented to permit the inclusion of unpublished data on the substrate specificity of the acid phosphatase preparations.

The results presented in Table II, taken from a single experiment, are representative of data from several experiments. It is interesting that the

TABLE I

Phosphatase Activity of Normal and Infected Tissue Preparations

	pH 4.6		pH 8.6	
	β-glycero-phosphate	Adenylic acid (yeast)	β-glycero-phosphate	Adenylic acid (yeast)
Normal allantoic fluid*	134	270	50	267
Infected allantoic fluid*	—	230	—	340
Normal chorio-allantoic membrane‡	240	360	200	314
Infected chorio-allantoic membrane‡	—	400	—	430
Normal mouse brain‡	—	430	—	340

* Values expressed as micrograms of PO_4 hydrolyzed in 4 hours per milliliter of fluid.

‡ Values expressed per milliliter of 10 per cent tissue suspension.

TABLE II

Effect of Phosphatase Inhibitors and Substrates on Virus Survival

	26 hrs. 37°C.	40 hrs. 37°C.
Virus titer "0" time	$10^{2.8}$	
Untreated virus (chorio-allantoic membrane suspension)	10^{1}	0
Virus + 0.1 M PO_4	$10^{2.3}$	0
DL-Alanine (0.06 M)*	$10^{2.6}$	$10^{1.5}$
DL-Lysine (0.06 M)	$10^{2.7}$	$10^{2.3}$
L-Arginine (0.06 M)	$10^{2.6}$	$10^{1.7}$
β-Glycerophosphate (2 mg.)	$10^{2.0}$	0
β-Glycerophosphate (10 mg.)	$10^{2.3}$	10^{1}
L-Histidine (0.06 M)	0	0
Glycine (0.06 M)	0	0
L-Tyrosine (0.06 M)	0	0
L-Tryptophane (0.06 M)	0	0
DL-Serine (0.06 M)	$10^{1.5}$	0

Titrated in eggs; three eggs per dilution.

* Molar concentrations expressed as final concentration. The values expressed are the number of infective units remaining per 0.05 ml. of undiluted material after indicated periods of incubation at 37°C. in contact with air.

basic amino acids are consistently more effective than others, an effect which is unrelated to pH as all samples were so buffered as to maintain effectively a pH of 7.0 to 7.2. Adenosine-3-phosphate showed the same protective action as β-glycerophosphate.

Action of Purified Alkaline and Acid Phosphatases.—After demonstration of

phosphatase activity in virus suspensions and of the greater survival of virus in the presence of phosphatase inhibitors, the action of phosphatases added to the system was tested.

A standard quantity of 0.1 ml. of phosphatase was added to 0.9 ml. of virus suspension plus 0.03 ml. of 4 per cent $MgCl_2$. To insure bacteriostasis penicillin was added to a final concentration of 500 units per ml. Hydrolysis was carried out at pH 7.3–7.6 in veronal acetate buffer for from 2 to 10 hours at 37°C. and samples assayed for residual infectivity.

Table III reports the finding that purified acid and alkaline phosphatases when added to virus suspensions did increase the rate of inactivation over the "normal." Magnesium alone did not affect the rate of virus inactivation.

TABLE III

Inactivation of Virus by Added Phosphatases

Virus and additions	Experiment 1 (CA)	Experiment 2 (A)	Experiment 3 (CA)	Experiment 4 (A)
Time incubated at 37°C., *hrs.*	6	4	10	8
Virus titer "0" time	$10^{4.2}$	10^{3}	$10^{4.2}$	$10^{4.5}$
Untreated	$10^{3.8}$	$10^{2.3}$	$10^{3.8}$	10^{4}
Acid phosphatase	$<10^{1}$	$<10^{1}$	$10^{1.5}$	$10^{2.5}$
Boiled acid phosphatase	$10^{2.9}$	$10^{1.6}$	$10^{3.8}$	—
Alkaline phosphatase	$<10^{1}$	—	—	$10^{1.6}$
Boiled alkaline phosphatase	—	—	—	$10^{3.3}$
Acid phosphatase + β-glycerophosphate (2 mg.)	10^{2}	$<10^{1}$	10^{2}	—
Acid phosphatase + L-arginine (0.12 M)	10^{3}	—	—	—

— indicates not done.
Titrated in eggs; three eggs per dilution.
Values expressed as infective units remaining.
CA, chorio-allantoic membrane suspension virus.
A, allantoic fluid virus.

It will be observed that boiling the phosphatase enzyme, a treatment considered to inactivate the enzyme, destroys most of the anti-viral activity of the preparation. What remains is probably non-specific since boiled or autoclaved preparations of serum, normal allantoic fluid, or tissue suspensions show similar slight anti-viral activity for herpes. The addition of substrate or amino acid inhibitors to the phosphatase-virus mixture partially prevented inactivation attributed to added phosphatase.

Inhibition of Purified Phosphatases by Cysteine and Fluoride.—Further confirmation of the specific activity of phosphatases in this attack upon virus by added enzyme came from the use of the selective inhibitors cysteine and fluoride (Table IV). Cysteine inhibits alkaline phosphatase and has no action against acid phosphatase, while fluoride behaves in just the opposite fashion (6). It was found that the addition of fluoride to phosphatase-virus mixtures

retarded the destruction by acid phosphatase but not by alkaline phosphatase as expected, while cysteine prevented the inactivation of virus by alkaline phosphatase but had no effect upon acid phosphatase. The control with cysteine underwent virtually no inactivation during the experimental period.

Enhancement of Cysteine Protection by Fluoride.—Cysteine, whose antioxidant effect (7) is difficult to dissociate from more specific effects, was found to exert a dramatic preservative action (Fig. 1). On the other hand 0.04 M sodium fluoride shows but slight preservative effect if added alone and sealed from air to parallel the procedure for cysteine-treated samples. The combina-

TABLE IV

Effect of Fluoride and Cysteine on Phosphatase-Virus Systems

Virus and additions	Experiment 1 (CA)	Experiment 2 (A)	Experiment 3 (CA)	Experiment 4 (CA)
Time incubated 37°C., *hrs.*	6	11	4	9
Virus titer "0" time	$10^{3.5}$	$10^{4.2}$	10^{3}	$10^{4.2}$
Untreated	10^{3}	$10^{2.9}$	10^{3}	10^{3}
Acid phosphatase	10^{2}	$<10^{1}$	$10^{2.2}$	10^{1}
Acid phosphatase + 0.02 M NaF	10^{3}	$10^{2.4}$	$10^{3.2}$	—
Acid phosphatase + 0.02 M cysteine	—	—	—	10^{2}*
Alkaline phosphatase	$10^{1.6}$	—	—	10^{1}
Alkaline phosphatase + 0.02 M NaF	$10^{1.8}$	—	—	—
Alkaline phosphatase + 0.02 M cysteine	—	—	—	$10^{3.8}$*
Cysteine (0.02 M)	—	—	—	$10^{4.2}$*

Titrated in eggs; three eggs per dilution.

—, indicates not done.

Values expressed as infective units remaining.

CA, chorio-allantoic membrane virus.

A, allantoic fluid virus.

* Cysteine added and sealed from air with mineral oil.

tion of cysteine and fluoride, however, results in an enhancement of the protection seen with cysteine alone. Only cysteine could be shown to inhibit the hydrolysis of adenosine-3-phosphate at pH 7.4 by endogenous phosphatase of infected allantoic fluids (Table V).

Inability to Demonstrate Formation of Viricidal Substances.—Experiments were designed to test the possibility that phosphatase action resulted in the production of a toxic material which in turn destroyed the virus. The techniques employed included: (*a*) addition of aliquots of tissue suspensions in which the virus had been enzymatically inactivated to fresh virus in varying proportions; and (*b*) the collection of dialyzable products in the course of enzymatic inactivation, concentration of the dialysate under partial vacuum at low temperature and the treatment of fresh virus with this material. In no

case could it be demonstrated that toxic substances were produced or that an inhibitor of viral infectivity was present.

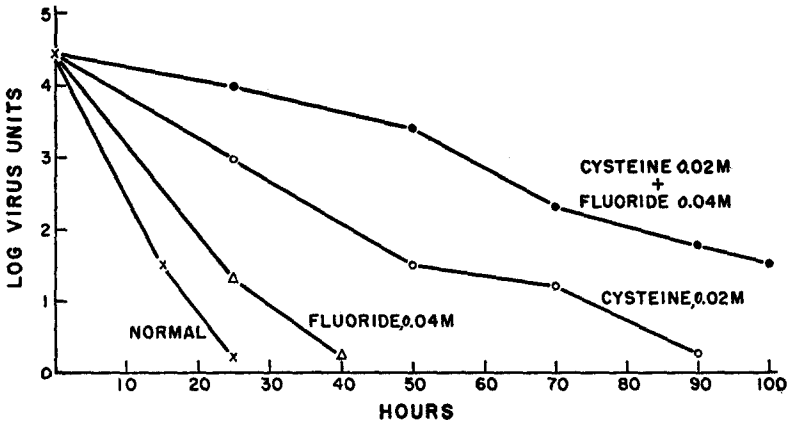

FIG. 1. Effect of fluoride and cysteine on survival of herpes simplex virus at 37°C. Residual infectivity titrated on chorio-allantoic membrane; 3 eggs per dilution.

TABLE V

Effect of Fluoride and Cysteine on Endogenous Phosphatase at pH 7.4

Tube	Inhibitor (final concentration)	Adenylic acid (yeast)	Phosphatase activity as μg. of phosphorus hydrolyzed (8 hrs. 37°C.)
		mg.	
1, control	0	0	*<1
2	0	15	29
3	0.01 M cysteine	15	17
4	0.01 M cysteine	0	<1
5	0.05 M fluoride	15	30
6	0.01 M fluoride	15	26
7	0.001 M fluoride	15	27
8	0.05 M fluoride	0	<1

Allantoic fluid was dialyzed at 4°C. against distilled water in motion for 12 hours and 2 ml. aliquots taken as enzyme for each tube.

* Limit of the method is 1 μg. These measures were made on the endogenous phosphatase activity of the allantoic fluid of chick embryos infected with herpes simplex virus 2 days before the fluids were harvested.

Effect of Other Enzymes on Viral Infectivity.—No inactivation of virus could be demonstrated on treatment with trypsin, pepsin, ribonuclease, ptyalin, or pancreatic lipase for varied intervals of time at pH 7.0–7.6.

DISCUSSION

The conclusion that phosphatase enzymes of the propagating tissue play a role in the inactivation of herpes simplex virus under the experimental con-

ditions here reported is based upon: (*a*) the relatively high level of phosphatase activity, both acid and alkaline, of the suspensions under study; (*b*) the increased survival of the virus in the presence of inhibitors of phosphatases and of phosphatase substrates; (*c*) the increase in the rate of inactivation following the addition of purified acid or alkaline phosphatase to the virus suspensions. This conclusion is drawn in full awareness that other factors contribute as well to the inactivation, some of which factors will be reported in a later publication.

Equimolar concentrations of the amino acids, DL-lysine and L-arginine, were consistently more effective in protecting viral infectivity against endogenous phosphatase than were DL-alanine, glycine, or L-histidine. This finding is not inconsistent with the types of differences Bodansky (6) has reported for the effectiveness of various amino acids as inhibitors of relatively crude intestinal, bone, and kidney phosphatases.

The marked protection rendered by cysteine and glutathione (unpublished data) is due probably in part to their anti-oxidant activity (7) as well as to their inhibition of endogenous alkaline phosphatase (8). Hyposulfite and an atmosphere of hydrogen or nitrogen, though protective (unpublished data), are much less effective than the sulfhydryl compounds. The failure of fluoride to prolong significantly the survival of infective virus when added alone may be attributable to a high level of alkaline phosphatase activity relative to that of acid phosphatase at pH 7.4 in these preparations. Indeed this hypothesis is consistent with the finding that the hydrolysis of adenosine-3-phosphate by endogenous phosphatase at pH 7.4 is unaffected by fluoride but inhibited by cysteine. We are uncertain to what degree the effect of these inhibitors in the hydrolysis of adenosine-3-phosphate resembles their influence on the breakdown of virus constituents and endogenous substrate.

The observation that phosphatase enzymes added to suspensions of the virus are capable of destroying its infectivity invites speculation as to the nature of this inactivation. Either it may be due to the production of a toxic substance that in turn inactivates the virus or the enzyme may destroy a component essential to viral infectivity. Attempts to demonstrate the production of an inactivating substance through enzymatic action on the virus or on tissue components proved unsuccessful. An essential component destroyed by enzymatic hydrolysis could be nucleic acid or a nucleic acid derivative or a molecule unrelated to nucleic acid. It will be recalled that the phosphatases employed as exogenous enzyme are able to split monoesters as well as diesters of phosphoric acid (4, 10). The alkaline phosphatase possesses ribonuclease activity but no desoxyribonuclease activity, while the acid phosphatase is contaminated with traces of ribonuclease. Long treatment, however, with recrystallized ribonuclease resulted in no harmful effect on the virus, which result tends to rule out ribonucleic acid as the component attacked by the phosphatases in their destruction of virus.

The simplest hypothesis is that some molecule essential to infectivity is active only in a phosphorylated form. We have found (unpublished data) that most of the inorganic phosphate that appears as a result of phosphatase action on these suspensions comes from the fraction containing largely adenosinediphosphate and adenosinetriphosphate (*i.e.*, the 7 minute hydrolyzable, barium-insoluble fraction). This, while it may prove of future significance, has little meaning now, since it need only be pointed out that most of the ADP and ATP is supplied by the host tissue and that much smaller quantities of some other molecule may be the crucial factor.

Attempts to reconstitute infectivity by the addition of ATP and synthetic acetyl phosphate to phosphatase-inactivated virus were unsuccessful; it is, therefore, unlikely that either of these two compounds is involved in a simple cofactor or energy-source function. The possibility persists that the substance required may be active only when bound to some other molecule and that this condition is not met by addition of free ATP or acetyl phosphate. Perhaps attention should be called to the inactivation of coenzyme A by intestinal phosphatase (8, 9). Both the ubiquitous distribution of coenzyme A in living cells and its linkage to a wide spectrum of transacetylating and transphosphorylating systems suggest investigation of its possible involvement in this system.

SUMMARY

Tissue phosphatases which are present in virus suspensions were found to contribute in considerable measure to the inactivation of herpes simplex virus.

Phosphatase inhibitors and phosphatase substrates have been found to prolong the survival of the virus.

Exogenous phosphatases, both acid and alkaline, have been demonstrated to bring about the loss of infectivity of suspensions of this virus.

I wish to express my sincere thanks to Professor J. Howard Mueller for his guidance and patient supervision of the thesis from which this material is taken.

BIBLIOGRAPHY

1. Burnet, F. M., and Beveridge, W. I. B., *Great Britain Med. Research Council, Special Rep. Series, No. 256*, 1946, 25.
2. Shaffer, M. F., and Enders, J., *J. Immunol.* 1939, **37**, 383.
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.
4. Schmidt, G., and Tannhauser, S. J., *J. Biol. Chem.*, 1943, **149**, 369.
5. Bodansky,. O, *J. Biol. Chem.*, 1948, **174**, 465.
6. Sumner, J. B., and Myrback, K., The Enzymes, New York, Academic Press, Inc., 1950, **1**, 483–489.
7. Barron, E. S. O., and Singer, T. P., *Science*, 1943, **97**, 358.
8. Lipmann, F., *J. Biol. Chem.*, 1945, **160**, 173.
9. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, 1948, **176**, 459.
10. Schmidt, G., unpublished data.