EFFECT OF PURIFIED ENZYMES ON VIRUSES AND GRAM-NEGATIVE BACTERIA

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(Received for publication, April 2, 1936)

Most of the work on the action of tryptic enzymes on viruses is difficult to interpret since the pancreatic extracts used contained substances other than the enzymes themselves. Pirie (1) has summarized the literature and from her own experiments suggests that the reported inactivation of viruses was due to the fatty acids and lecithins present in the extracts rather than to the enzymes. She was able to inactivate certain viruses by the fatty acids obtained from pancreatic extracts.

Pirie (1) found that crystalline trypsin and chymotrypsin had no effect, or at most a very slight one, on pleuropneumonia, vaccinia, and fowl tumor viruses under the conditions of her experiments. Stanley (2), using crystalline trypsin, found that the reported action of trypsin on tobacco mosaic virus was due to an effect on the host and not on the virus. He further found (3) that this virus was inactivated by pepsin. This last enzyme cannot be used with many animal viruses, since the acidity at which it is active quickly destroys them. Bawden and Pirie (4) have reported the inactivation of the S strain of potato virus, as well as of its power to react with antiserum, by crystalline preparations of trypsin and pepsin.

Since proteolytic enzymes have been obtained in a pure form by Northrop and Kunitz (5), it is now possible to determine more accurately whether viruses are susceptible to their action. The subject is of importance, for if viruses can be inactivated by proteolytic enzymes, it would indicate that they are protein in nature. It would suggest furthermore that they are not living agents, since, according to Northrop (6), all known living cells resist tryptic digestion. It is our purpose to report here experiments made with crystalline trypsin and chymotrypsin on four animal viruses and on a number of Gram-negative bacteria.

Materials and Methods

Drs. Northrop and Kunitz kindly supplied preparations of trypsin and chymotrypsin that had been repeatedly crystallized and then dissolved in sufficient buffer to give an approximate concentration of 1 mg. nitrogen per cc. The proenzyme chymotrypsinogen, similarly purified, was used as a control and also after it had been changed to chymotrypsin. The action of these enzymes has been tested on the viruses of equine encephalomyelitis, pseudorabies, swine influenza, and vaccinia.

Since we knew that the first two viruses mentioned are rapidly inactivated at incubator temperature, and since this inactivation can be slowed down by excluding air, all mixtures were kept at refrigerator temperature under vaseline seal. The following procedure was used throughout the work: All solutions were cooled to the temperature of the ice bath before mixtures were made. 5 cc. of the suspension containing virus was mixed with 4.5 cc. of the enzyme solution and 0.5 cc. of 4 per cent sterile neutral cysteine hydrochloride, and melted sterile vaseline was placed on the surface of the mixture. In all experiments except those recorded in Text-fig. 1 and Table II, the final concentration of the enzymes in the mixtures was approximately 0.5 mg. enzyme N per cc. Dilutions were made in salt solution kept at the temperature of an ice bath and inoculations were made immediately after finishing the dilutions.

The stock 4 per cent aqueous solution of cysteine hydrochloride was prepared and adjusted to a pH of 7.3–7.4, passed through a Berkefeld filter, and the filtrate distributed into test tubes and sealed with vaseline. The physiological salt solution used was buffered by adding to each 100 cc., 2 cc. of a stock buffer solution containing 28.81 gm. of NaH_2PO_4 and 125 gm. of Na_2HPO_4 per liter. Tests demonstrated that the cysteine hydrochloride did not inhibit the action of trypsin upon casein.

The activity of the enzyme was always demonstrated by its action on casein and gelatin at the end of the experiment. In all experiments the pH was determined at the beginning and end in order to be sure that inactivation of the viruses was not due to a change in reaction. No marked change occurred.

Since enzyme action is markedly slowed at low temperatures, the mixtures were kept for a much longer period of time than would ordinarily be used in digestion experiments. That the enzymes were active under the conditions was repeatedly demonstrated by their action on gelatin and casein.

The criteria for action of the enzyme on the virus were:

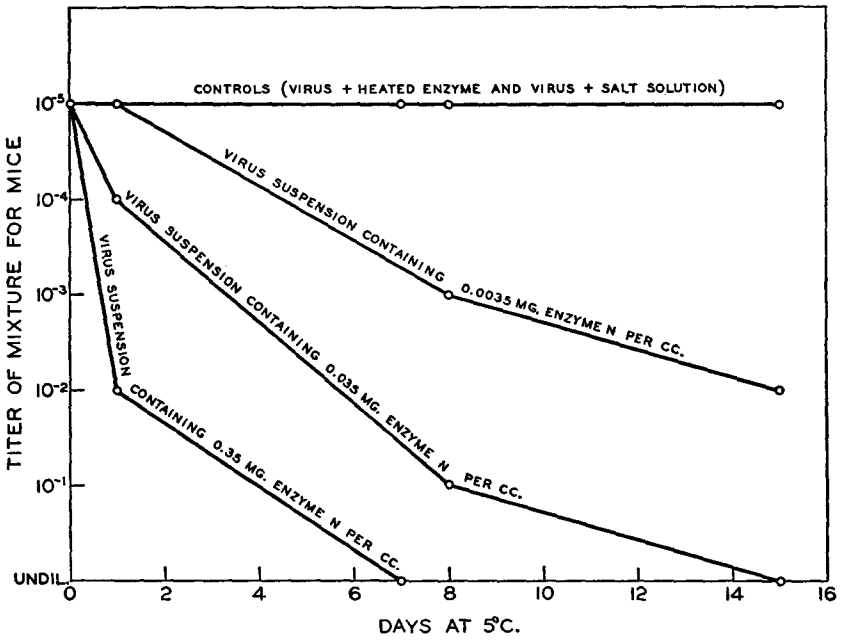
1. No immediate inactivation of the virus but inactivation after storage, the degree depending on the length of time stored.

2. Within a given time, the greater the concentration of the enzyme, the more inactivation.

3. No inactivation by chymotrypsinogen nor by boiled trypsin or chymotrypsin.

Effect on Equine Encephalomyelitis Virus

An Eastern strain of equine encephalomyelitis virus isolated in this laboratory was used. Guinea pigs or mice were inoculated intracerebrally, and immediately after death their brains were ground and a 10 per cent suspension made in saline. This suspension was distributed into test tubes, sealed with vaseline, and stored in the refrigerator. The supernatant obtained after centrifugalizing the suspension was mixed with the enzymes and tested by intracerebral injection into guinea pigs as given in the tables.



TEXT-FIG. 1

In Text-fig. 1 are the results of tests made on uniform quantities of virus to which were added decreasing amounts of a mixture of trypsin and chymotrypsin. It will be seen that, judged by the criteria given, the virus was destroyed by the mixture of the two enzymes. Subsequent tests with various preparations of trypsin showed that this enzyme failed to inactivate the virus but that chymotrypsin did so. In Table I are the results of such an experiment. It should be pointed out that the chymotrypsin was prepared from the same lot of chymotrypsinogen used in this experiment by the addition of a small amount of the same trypsin found to be inactive.

In Table II are results which show that the degree of inactivation by chymotrypsin is dependent upon the amount of the enzyme present.

TABLE I

Effect of Enzymes on Equine Encephalomyelitis Virus

Enzyme added	Time after mixing when tested	Dilution of mixture inoculated 0.03 cc. intracerebrally into mice				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Chymotrypsinogen	Immediately				++	++
	4 days	++	++	++	++	+0
Chymotrypsin	Immediately				++	++
	4 days	++	+0	00	00	00
Trypsin	Immediately				++	++
	4 days			++	+0	++
Chymotrypsin (heat-inactivated) (control)	Immediately				++	00
	4 days			++	++	++
None (control)	Immediately				++	++
	4 days			++	++	++

Each + indicates a mouse that died, and each 0 a mouse that survived.

TABLE II

Effect of Decreasing Amounts of Chymotrypsin on Equine Encephalomyelitis Virus after 6 Days' Storage

Dilution tested	Amount of enzyme N per cc.				Salt solution plus virus (control)
	0.4 mg.	0.04 mg.	0.004 mg.	0.4 mg. boiled	
10^{-1}	*	++	++		
10^{-2}	00	+0	++		
10^{-3}	00	00	++	++	++
10^{-4}	00	00	+0	++	++
10^{-5}	00	00	00	++	00

Each + indicates a mouse that died, and each 0 a mouse that survived.

* Died immediately from inoculation.

Effect on Pseudorabies Virus

The brain of a rabbit infected with the Aujeszky strain of pseudorabies was suspended and stored in the same way as the equine enceph-

alomyelitis virus. The activity of the mixtures was tested by either subcutaneous or intracerebral inoculation of guinea pigs. In Table III the results of one of the tests are given. They show that both chymotrypsin and trypsin inactivate this virus. This inactivation by both enzymes has been repeatedly obtained after storage of the mixtures, whereas immediately after mixing the virus is found to be active.

Effect on Swine Influenza Virus

A suspension of lungs from mice infected with swine influenza was obtained from Dr. Shope. White mice were anesthetized and their noses were immersed in this suspension for approximately 6 seconds. The animals inoculated in this way either died or were very sick by the 4th day, when their lungs were removed and a 5 per cent suspension of the pulmonary tissue in salt solution was prepared. This suspension was centrifugalized and to the supernatant were added the various enzymes. After 5, 10, and 28 days' storage in the refrigerator samples were withdrawn, diluted, and two mice inoculated with each dilution, in the same manner used for the preparation of the virus.

TABLE III

Effect of Enzymes on Pseudorabies Virus When Tested after 7 Days' Storage at 5°C.

Dilution of mixture*	Result of intracerebral injection of guinea pigs with virus plus				
	Chymotrypsinogen	Chymotrypsin	Trypsin	Heated chymotrypsin (control)	Salt solution (control)
1:5		0	0		
1:25		0	0		
1:125	+	0	0	+	+
1:250	+	0	0	+	+
1:500	0	0	0	0	0
1:1000	+	0	0	0	+

0 = survived. + = died.

*A 5 per cent suspension of brain from an infected rabbit was used in this experiment.

The results of the one test made are given in Table IV, and it will be seen that there is no indication of destruction of the virus by the various enzymes used. There is a considerable loss of titer after 28 days' storage, but the loss in the control tubes is the same as in those containing active enzymes.

TABLE IV

Effect of Enzymes on Swine Influenza Virus

Swine influenza virus plus	Result of intranasal inoculation of mice with mixtures kept at 5°C. for																	
	5 days*						10 days†						28 days‡					
	Dilution of mixtures inoculated																	
	1:5	1:25	1:125	1:625	1:3125	1:9375	1:5	1:15	1:45	1:135	1:405	1:1215	1:5	1:25	1:125	1:625	1:1250	1:2500
Chymotrypsinogen	++	+	+	+	0	0	D 6	D 7	D 8	D 9	++	+	D 8	++	0	0	0	0
	++	++	++	+	0	0	D 8	D 7	D 8	+++	++	+	+	+	++	0	0	0
Chymotrypsin	+++	+	++	0	0	0	D 7	D 8	D 9	++	++	+	+	++	+	+	0	0
	++	+	+	0	0	0	D 9	++	++++	+	+	0	+++	+++	+++	0	0	0
Trypsin	+	0	0	+	0	0	D 7	D 8	D 9	++	0	++	D 6	++	+	0	0	0
	++	+++	++	++	0	0	++++	D 9	D 8	+++	+	++	++	+	++	0	0	0
Heated chymotrypsin (control)	+++	++	+	++	0	0	D 3	D 4	D 7	D 10	++	++	++	D 7	D 7	0	0	0
	++	++++	+	++	0	0	D 5	D 6	D 7	+++	++	++++	++	+++	++	0	0	0
Salt solution (control)		+++	+++	+	0	0	D 6	D 8	D 9	++	+	+++	+++	++	+++	0	0	0
		+++	++	++	0	0	D 8	D 8	++++	++	+++	+++	+++	++	0	0	0	0

D 6 = dead 6 days after inoculation.

Each + indicates the involvement of one lobe of the lung.

* Autopsies 4 days after inoculation.

† " 10 " " "

‡ " 14 " " "

TABLE V

Effect of Proteolytic Enzymes on Washed Vaccinia Elementary Bodies

Dilution of mixture tested	Tested after, *days*										
	0	7	13	20	0	7	13	20	7	13	20
	Chymotrypsin				Trypsin				Heated trypsin (control)		
10^{-1}	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
10^{-2}	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
10^{-3}	++++	++++	++++	++++	++++	++++	++++	++	++++	+++	++++
10^{-4}	++++	++++	++++	+++	++++	+++	++	0	+++	++	++
10^{-5}	+++	+++	+++	++	+++	++	+	0	++	+	+
10^{-6}	++	++	+	+	++	0	0	0	0	0	0
10^{-7}		+	0	0		0	0	0	0	0	0

Dilution of mixture tested	Tested after, *days*										
	0	7	13	20	7	13	20	0	7	13	20
	Trypsin-chymotrypsin				Heated trypsin-chymotrypsin (control)			Salt solution (control)			
10^{-1}	++++	++++	++++	++	++++	++++	++++	++++	++++	++++	++++
10^{-2}	++++	++++	++	±	++++	++++	++++	++++	++++	++++	++++
10^{-3}	++++	+++	++	0	++++	++++	++++	++++	++++	++++	++++
10^{-4}	++++	++	+	0	++++	++++	++++	++++	+++	+++	+++
10^{-5}	+++	+	0	0	+++	+++	+++	+++	+++	++	++
10^{-6}	++	0	0	0	++	++	++	++	++	+	+
10^{-7}		0	0	0	±	+	±		±	0	0

Effect on Vaccinia Virus

In the first two experiments a suspension was used of the testicles of a rabbit inoculated with the New York State Board of Health strain of vaccinia virus. There was no definite evidence of inactivation by either trypsin or chymotrypsin, the mixtures being kept for 3 weeks under the conditions described above.

A test was then made using a washed suspension of elementary bodies obtained from Dr. T. M. Rivers. This suspension was diluted 1:5, and 0.5 cc. of ether was added to each 10 cc. of suspension. Equal amounts of this suspension and of the enzyme preparations were mixed and tested by intracutaneous inoculation into rabbits, as given in Table V. The results show no inactivation by chymotrypsin, slight inactivation by trypsin, and a more pronounced inactivation by a mixture of equal parts of trypsin and chymotrypsin.

TABLE VI

Effect of Trypsin on Gram-Negative Bacilli

Bacterium tested	Bacterial count after 10 days at 5°C.	
	In the presence of trypsin	Control
B. bovisepticus	1.0×10^8	2.5×10^8
B. coli II (non-motile)	5.9×10^8	2.7×10^8
B. coli III (motile)	9.9×10^8	9.2×10^8
B. enteritidis	1.6×10^9	1.2×10^9
B. paratyphosus A	3.7×10^8	2.2×10^8
B. paratyphosus B	4.3×10^8	3.8×10^8
B. pseudotuberculosis	4.8×10^8	2.7×10^8
B. pullorum	1.5×10^9	2.1×10^9
B. suipestifer	2.0×10^8	1.9×10^8
B. typhosus	9.0×10^7	9.0×10^7

Action on Gram-Negative Bacteria

In the older literature (7) it is stated quite definitely that enzymes do not digest living Gram-negative bacteria, but recent papers by Bruner (8) and by Day and Gibbs (9) raise the question of whether proteolytic enzymes do not after all digest the organisms. Because of these recent papers and also because there are no reports on the action of purified enzymes on bacteria, it seemed advisable to restudy this subject.

In Table VI are the results of an experiment on the effect of trypsin on ten different Gram-negative bacteria. The conditions were the

same as with the viruses, and it will be seen that under these conditions there was no destruction of any of the organisms. Other experiments, which need not be given in detail, were made with chymotrypsin and trypsin, and in mixtures kept up to 30 days there was no evidence of digestion of the organisms. When added to nutrient broth either trypsin or chymotrypsin apparently increased the growth of every organism tested. Killed Gram-negative bacteria were rapidly digested by either enzyme, while Gram-positive organisms, either living or dead, resisted the action of both enzymes.

DISCUSSION

The results given show that viruses differ in their resistance to inactivation by tryptic enzymes. Equine encephalomyelitis virus is inactivated by chymotrypsin and not by trypsin; vaccinia is slowly inactivated by trypsin, but chymotrypsin does not affect its activity; pseudorabies virus is inactivated by both enzymes; and swine influenza virus is inactivated by neither.

Although not recorded in the tables, it was shown in every case in which viruses were inactivated that the inactive mixtures when mixed with fresh virus did not influence the strength of the latter. This is evidence that the digestion products were not responsible for the inactivation. Since the viruses were not in a pure state it is impossible to say that inactivation and digestion are the same, but it is possible to say that some viruses behave like proteins in that they are susceptible to the action of proteolytic enzymes.

The fact that some viruses are not inactivated by the enzymes used does not rule out the possibility that they too may be protein in nature. If we had other enzymes it might be found that these resistant viruses were inactivated by them, or it may be that, like killed Gram-positive bacteria, they resist the action of the trypsins.

The bacteria tested behave like other known living organisms in that they resist digestion with tryptic enzymes. Some of the viruses are inactivated, on the other hand. This can be taken as presumptive evidence that the viruses are non-living. In this connection one may recall the fact that Stanley (10) has isolated a crystalline protein which has the properties of tobacco mosaic virus.

The findings suggest the possibility of classifying viruses according

to their resistance to trypsin and other enzymes. Such a classification would be useful when it is determined how these various enzymes differ in their action, for it might throw light on the structure of the viruses.

SUMMARY

Evidence is presented that some viruses behave like proteins in that they are inactivated by proteolytic enzymes, whereas others prove more or less resistant. Ten strains of living Gram-negative bacteria resisted the action of purified trypsin and chymotrypsin, while the killed organisms were rapidly digested. Gram-positive bacteria, on the other hand, were resistant whether living or dead. The findings are discussed.

Drs. Northrop and Kunitz not only supplied the enzyme preparations but were generous in giving advice and criticism.

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