INHIBITION OF THEILER'S ENCEPHALOMYELITIS VIRUS (GDVII STRAIN) OF MICE BY AN INTESTINAL MUCOPOLYSACCHARIDE

I. Biological Properties and Mechanism of Action*

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Following the observation that influenza viruses can agglutinate erythrocytes from certain animal species (1), numerous reports have appeared in which various substances are described that are capable of inhibiting hemagglutination (2–16). It has also been shown (6, 9, 15–20) that under suitable conditions influenza viruses are able to destroy the inhibitory property of these substances. These naturally occurring inhibitors of hemagglutination do not possess comparable inhibitory activity with respect to the infectivity of virus suspensions.

In contrast to inhibitors of hemagglutination, it has been shown (21–23) that the capsular polysaccharide of Type B Friedländer bacilli is capable of inhibiting the multiplication of mumps virus and pneumonia virus of mice (PVM). Studies on the inhibition of viral multiplication by this polysaccharide have shown that there is no interaction between virus and bacterial polysaccharide (24), but rather that the inhibitor affects the host (25, 26).

A third type of viral inhibition has been described by Levine and Frisch (27) who showed that extracts of bacteria susceptible to certain bacteriophages inhibited the lytic action of these viruses. Further studies of this phenomenon by Miller and Goebel (28) revealed that in the case of Phase II *Shigella sonnei*, the carbohydrate haptene of the somatic antigen reacts with the bacteriophage and inhibits its infectivity.

In a preliminary note (29) it was reported that a substance obtained from the intestines of mice inhibits both hemagglutination and infectivity by the GDVII strain of Theiler's encephalomyelitis virus of mice. The present paper will describe these studies in greater detail. In common with the influenza virus inhibitors, the intestinal inhibitor prevents viral hemagglutination, and

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in common with the mumps-PVM and bacterial virus inhibitors, it reduces infectivity of GDVII virus.

The effects observed *in vivo* and *in vitro* appear to result from interaction between virus and inhibitor. Finally, an enzyme present in the feces of mice has been shown to be capable of destroying the activity of the inhibitor with a concomitant release of reducing sugars.

In the accompanying paper (30) evidence is presented which indicates that the inhibitor is a mucopolysaccharide.

Materials and Methods

Viruses[1].—The following viruses were used: GDVII, FA, and TO strains of Theiler's mouse encephalomyelitis virus; Lansing (mouse-adapted) strain of poliomyelitis virus. A distinction is made between TO virus obtained directly from the feces or the intestines of mice, and TO virus obtained after passage in the central nervous system (CNS) of the mouse, by designating the former TO(I) and the latter TO(B).

TO(I) virus was obtained from feces or small intestines of 4 to 6 week old mice. The feces or small intestines were homogenized with 10 volumes of distilled water. After centrifugation at 2500 R.P.M. for 15 minutes, the supernatant liquid was decanted, mixed with approximately one-half its volume of ether, and allowed to remain overnight in the refrigerator. The aqueous phase was then collected and the residual ether was removed by means of reduced pressure. After centrifugation at 2500 R.P.M. for 15 minutes, the supernatant liquid was decanted and stored.

GDVII, FA, TO(B), and Lansing viruses were propagated by CNS passage in mice. In preparing the suspensions of GDVII and FA viruses, the brains of infected mice were removed and homogenized with 10 volumes of distilled water. The TO(B) and Lansing viruses were prepared in the same manner with the exception that both brains and spinal cords were harvested.

Virus preparations were stored at −70°C. in a dry-ice chest.

Mice.—The mice used in these experiments were the CFW strain obtained from Carworth Farms, New City, New York.

Virus Titrations.—The infectivity titers of GDVII and FA viruses were determined by the method of Gard (31) and expressed as $1/T$ value. Serial dilutions of extracts of infected mouse brain were prepared in cold saline solution (0.85 per cent NaCl). For each virus dilution six lightly anesthetized mice 4 weeks of age were inoculated intracerebrally with 0.03 ml. The mice were observed daily for characteristic symptoms. The $1/T$ values were computed from the equation (31):—

$$1/T = \frac{1/t_1 + 1/t_2 + \cdots 1/tN}{N}$$

in which $t_1 \ldots tN$ are the incubation periods in individual mice and N is the total number of inoculated mice. Three titration curves for GDVII virus are shown in Fig. 1.

It can be seen that there is a satisfactory linear relationship between the $1/T$ values and the logarithms of the dilutions between 10^{-3} and 10^{-6}. These

[1] GDVII virus was obtained from Dr. M. Theiler of The Rockefeller Institute for Medical Research; FA and Lansing viruses were obtained from Dr. R. Ward, New York University College of Medicine.

results are not entirely in agreement with those of Gard (31) who reported proportionality over the entire range of dilutions. This disagreement may be due to differences in virus or mouse strains employed. In this laboratory it was found that with low dilutions of the virus (*e.g.* 10^{-1}, 10^{-2}), the $1/T$ values were not accurate measures of virus titer since all the test mice either showed signs of infection or were dead on the 2nd day after inoculation. As a rule, $1/T$ values below 0.10 were found to be unreliable.

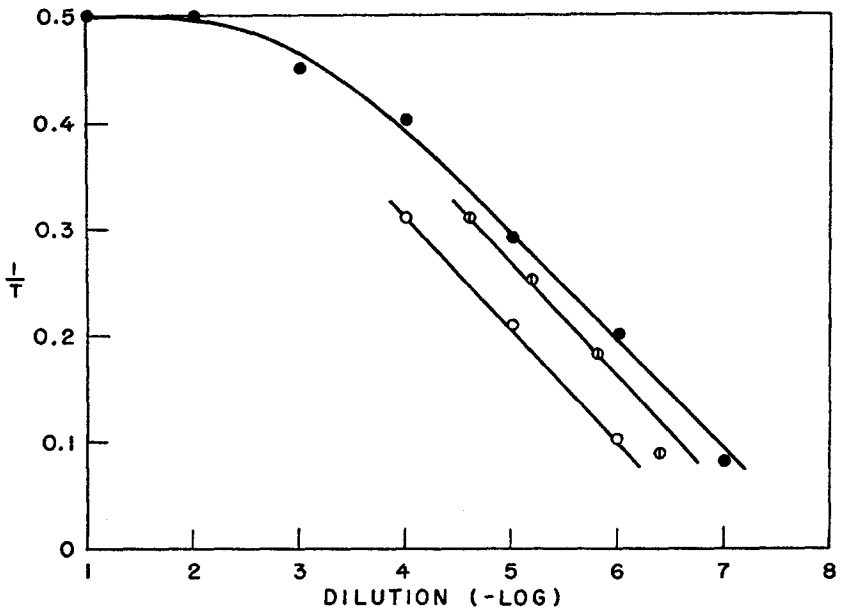

FIG. 1. Relation between $1/T$ value and logarithm of dilution of virus. Each curve was obtained with a different viral preparation (●, ○, ⦶). Each point represents the $1/T$ value for a group of 6 mice.

The hemagglutinin titer of GDVII virus was determined by a modification of the method of Lahelle and Horsfall (32). Human group O red blood cells (RBC) were collected aseptically in an equal volume of sterile Alsever's solution and kept in the refrigerator as a stock suspension. As needed, a portion of the cells was washed with saline solution and a 0.4 per cent suspension in saline was prepared. Serial twofold dilutions of the virus were prepared in 0.5 ml. of saline in 10 by 75 mm. tubes. 0.5 ml. of 0.4 per cent RBC was added and the tubes kept for 2 ½ hours in an icebath. The titer of the virus was taken as the highest dilution of the virus which caused complete agglutination of the RBC. This dilution was said to contain 1 hemagglutinating unit (HU) per 0.5 ml.

The infectivity titers of TO(I), TO(B), and Lansing viruses were determined by the 50 per cent end-point method of Reed and Muench (33).

Inhibitor Titrations.—The inhibitor was titrated *in vivo* by estimating its inhibitory effect on the infectivity of the virus as indicated by the reduction in the $1/T$ value. *In vitro*, the inhibitor was titrated by its inhibitory effect on viral hemagglutination.

In titrating the inhibitor *in vivo*, equal volumes of a solution of inhibitor and of virus, diluted so as to have a $1/T$ value of about 0.30, were mixed and 0.03 ml. inoculated intracerebrally into each of 6 mice. A virus control was prepared by substituting saline for the inhibitor. From the virus titration curves (Fig. 1), it can be seen that a change of 0.10 in $1/T$ corresponds to a change of 1 log unit in concentration of virus. On the basis of this relationship, inhibitory activity was estimated by the effect on the $1/T$ value.

In vitro titration of the inhibitor was carried out by preparing serial twofold dilutions of the inhibitor in 0.3 ml. of saline. To each dilution, 0.2 ml. of virus containing 8 HU was added and after 30 minutes in an icebath, 0.5 ml. of erythrocytes (0.4 per cent) was added. Appropriate virus and erythrocyte controls were included. The tubes were kept for 2 ½ hours in an icebath. The end-point was taken as the highest dilution of inhibitor capable of causing complete inhibition of hemagglutination. This dilution was said to contain 1 hemagglutination inhibition unit (HIU) per 0.3 ml.

Preparation of Acetone-Dried Powder from Tissues.—The tissue was excised from the animal and minced well with scissors. Stomach and intestines, however, were first cleaned either by slitting open and washing out the contents, or by gently squeezing out the contents. The minced tissue was homogenized thoroughly in a Waring blendor at room temperature with 10 volumes of acetone and centrifuged at 2000 R.P.M. for 15 minutes. The supernatant fluid was discarded and the sediment was washed successively with 10 volumes of acetone and 10 volumes of ether and finally dried *in vacuo*. If the acetone-dried powder was not extracted immediately, it was stored in a desiccator.

Each acetone-dried powder was assayed for inhibitor content by extracting it with 10 volumes of saline for 1 hour at 37°C. The suspension was centrifuged and the supernatant fluid was titrated *in vivo* and *in vitro* for inhibitory activity.

RESULTS

Demonstration of an Inhibitor of GDVII Virus in Intestinal Tissue of Mice.—In the course of studies on the interaction between tissues obtained from mice and several neurotropic viruses, it was observed that the titer of infectivity of Theiler's GDVII mouse encephalomyelitis virus was markedly reduced following contact with washed, minced intestine from adult mice. A similar effect on viral titer was observed in the presence of brain tissue but it was less pronounced and not as reproducible as with intestinal tissue. The experiments were carried out by adding the virus to the washed, minced tissue, incubating for 1 hour at 5°C., centrifuging, and assaying the cell-free supernatant fluid for viral content. The reduction in infectivity titer of the supernatant fluid suggested the possibility that virus had been adsorbed to the tissue mince. Attempts to recover the virus from the tissue particles by elution were unsuccessful, however.

A reduction in titer also occurred when virus was mixed with an aqueous extract of the intestinal tissue. The chemical properties of this water-soluble inhibitory substance were investigated for the purpose of developing a method of purification. It was found that when intestinal tissue was extracted with acetone and ether and dried, the inhibitor remained in the residue. An aqueous extract of this residue lost little or no activity when heated for 10 minutes at 100°C. or when dialyzed against distilled water. Neither trichloracetic acid nor shaking with chloroform precipitated or inactivated the inhibitor.

Purification of the GDVII Virus Inhibitor.—Based on the above findings a method for the purification of the inhibitor was developed. The method, described in detail in the following paper (30), involves the removal of lipids by organic solvents, precipitation and denaturation of proteins by trichloracetic acid and shaking with chloroform, precipitation of nucleic acids with $CuSO_4$, and finally the precipitation of the inhibitor with ethanol. On the basis of the analytical data (30), the inhibitor has been identified tentatively as a mucopolysaccharide.

TABLE I

GDVII Virus Inhibitor Content of Various Tissues of Adult Mice

Tissue	Hemagglutination-inhibition	Infectivity-inhibition		
		Dilution	$\frac{1}{T}$	Virus control, $\frac{1}{T}$
	HIU/gm. dry weight			
Brain and Cord	5,000	1:40	0.22	0.24
Lung	5,000	1:40	0.17	
Heart	2,500	1:40	0.25	
Spleen	<300	1:40	0.23	
Kidney	5,000	1:40	0.21	
Liver	5,000	1:40	0.23	
Muscle	2,500	1:40	0.24	
Stomach	85,000	1:40	0.11	
		1:400	0.21	
Small intestine	170,000	1:40	0	
		1:400	0	
Large intestine	2,500	1:40	0.19	

Biological Activity.—By the *in vivo* method it was found that 0.03 μg. of the purified inhibitor reduces the $1/T$ value of the virus from 0.30 to between 0.20 and 0.15. This reduction represents neutralization of 25 to 30 LD_{50} of virus. *In vitro*, 0.03 to 0.06 μg. inhibits 8 hemagglutinating units of GDVII virus.

Distribution of the Inhibitor among Various Tissues of Adult Mice.—A study was undertaken to determine the inhibitor content of various tissues of adult mice.

Several mice, approximately 4 months old, were sacrificed, the following organs excised, and an acetone-dried powder of each prepared: brain and spinal cord, lung, heart, spleen, kidney, liver, muscle, stomach, small intestine, and large intestine. Each powder was extracted and the individual extracts assayed for inhibitory activity. The results are recorded in Table I.

It is clear from the data in Table I that the inhibitory activity of the small intestine and stomach greatly exceeds that of any of the other specimens examined. Although the stomach extract contained about one-half as much inhibitor as the extract of small intestine as measured by hemagglutination inhibition, the intestinal extract was at least 10 times more active in inhibiting

infectivity. Further studies were therefore carried out only with the intestinal extracts.

Comparison of the GDVII Virus Inhibitor Content of the Intestinal Tissue of Adult and Infant Mice.—Olitsky (34) found that while he could recover TO virus from the intestinal tract of adult mice with great regularity, he was never able to demonstrate this virus in the intestinal tract of mice younger

TABLE II

Comparison of GDVII Virus Inhibitor Titers Found in the Intestinal Tissue of Infant and Adult Mice

Experiment No.	Age group	Hemagglutination-inhibition	Infectivity-inhibition		
			Dilution	$\frac{1}{T}$	Virus control, $\frac{1}{T}$
		HIU/gm. dry weight			
1	Infant				
	Pool 1	3,000	1:40	0.17	0.29
			1:400	0.26	
	Pool 2	1,600	1:40	0.11	
			1:400	0.22	
	Adult	50,000	1:40	0.11	
			1:400	0.09	
2	Infant				
	Pool 1	6,000	1:40	0.04	0.28
			1:400	0.17	
	Pool 2	6,000	1:40	0.06	
			1:400	0.20	
	Adult	160,000	1:40	0.08	
			1:400	0.08	

than 12 days of age. Because of this difference it was thought to be of interest to examine infant as well as adult mouse intestinal tissue for content of inhibitor.

Intestines from mice ranging in age from 5 to 8 days were pooled and acetone-dried powders prepared. Extracts of these powders were compared with extracts of acetone-dried powders of adult intestinal tissue.

The results of this study which are recorded in Table II, show that the intestinal tissue of adult mice contains at least 10 times as much inhibitor on a dry weight basis as the intestinal tissue of infant mice.

Since these 2 groups of mice differ in respect to both age and presence of TO virus in the intestinal tract, a similar study was carried out with adult mice from a Theiler-free mouse colony (29) and adult mice from the regular stock which are intestinal carriers of TO virus. It was found that the intestines of the Theiler-free mice contain inhibitor in somewhat higher concentration than the intestines of the virus-bearing mice.

It can therefore be stated that the presence of a high concentration of inhibitor in the intestines is independent of the presence of TO virus. There is, moreover, a suggestion that TO virus in the intestinal tract is associated with a slight reduction in the inhibitor content.

The GDVII Virus Inhibitor Content of the Intestinal Tissue of Various Species of Animal.—A survey was undertaken to determine whether the intestinal tissue of any species of animal other than the mouse contains the GDVII virus inhibitor.

Acetone-dried powders were prepared of the small intestine of the following animals: man, monkey, rat, cotton rat, hamster, guinea pig, rabbit, sheep, cow, and pig. The powders were extracted and each extract assayed.

The data in Table III show that only guinea pig intestinal tissue contains an inhibitor for GDVII virus in quantity similar to that found in the intestines of mice. No attempts have as yet been made to characterize this inhibitor from guinea pig.

The Mechanism of the Inhibition Reaction.—To explain the nature of the hemagglutination-inhibition reaction, 2 hypotheses were considered: (*a*) virus and inhibitor react to form an inactive complex; (*b*) inhibitor reacts with erythrocytes in such a manner as to render them inagglutinable by virus.

To test the latter hypothesis, equal volumes of an inhibitor solution (1 μg./ml.) and a 10 per cent red cell suspension were mixed and placed in an icebath for 2 hours. The mixture was then centrifuged and the supernatant fluid removed as completely as possible. The erythrocytes were resuspended in saline to a concentration of 0.4 per cent and used in titration of a GDVII virus preparation. The supernatant fluid was titrated for inhibitor content. The results are shown in Table IV.

The data in Table IV show that under the conditions employed in the hemagglutination-inhibition test, the erythrocytes failed to adsorb any detectable amounts of inhibitor, nor were the erythrocytes affected in their capacity to be agglutinated by GDVII virus. This observation is incompatible with the hypothesis that inhibition of hemagglutination is the result of a reaction between inhibitor and red cells.

The possibility that virus and inhibitor interact was examined by studying the effect of varying the period of incubation of the virus-inhibitor mixture prior to the addition of the red cells. The results of this study are depicted graphically in Fig. 2 in which the titer of hemagglutination-inhibition is plotted

TABLE III

GDVII Virus Inhibitor Content of the Small Intestine of Animal Species

Experiment No.	Species	Hemagglutination-inhibition	Infectivity-inhibition		
			Dilution	$\frac{1}{T}$	Virus control, $\frac{1}{T}$
		HIU/gm. dry weight			
1	Man	500	1:40	0.28	0.29
	Monkey	<500	1:40	0.26	
	Rat	1,000	1:40	0.23	
	Mouse	26,000	1:200	0.06	
			1:2000	0.21	
2	Cotton rat	2,600	1:40	0.23	0.28
	Hamster	*	1:40	0.22	
			1:400	0.28	
	Guinea pig	53,000	1:40	0.03	
			1:400	0.12	
	Mouse	53,000	1:40	0.11	
			1:400	0.09	
3	Rabbit	2,600	1:40	0.21	0.24
	Sheep	1,300	1:40	0.35	
	Mouse	22,000	1:40	0.10	
			1:400	0.10	
4	Cow	1,600	1:40	0.32	0.31
	Pig	1,600	1:40	0.32	
	Mouse	51,000	1:40	0.14	
			1:400	0.11	

* RBC pattern was atypical and an end-point could not be read unequivocally.

as a function of time. It can be seen that the end-point varies with the length of time that the virus-inhibitor mixture is held at 5°C. before addition of the red cells. The finding of such a dependence on time indicates an interaction between virus and inhibitor. Virus controls in the absence of inhibitor retained their titers undiminished throughout the period of the experiment.

The nature of the inhibition of viral infectivity in mice was investigated by varying the order of intracerebral inoculation of virus and inhibitor and the time intervals between the 2 inoculations. When the first inoculum was inhibitor, the mice to be used to test the virus controls were injected with saline.

TABLE IV

Failure of Erythrocytes to Adsorb the GDVII Virus Inhibitor

	Inhibitor titer*		Hemagglutinin titer*
Inhibitor pretreated with erythrocytes	8	Erythrocytes pretreated with inhibitor	160
Inhibitor control	8	Erythrocyte control	160

* Titers are expressed as the reciprocal of the dilution.

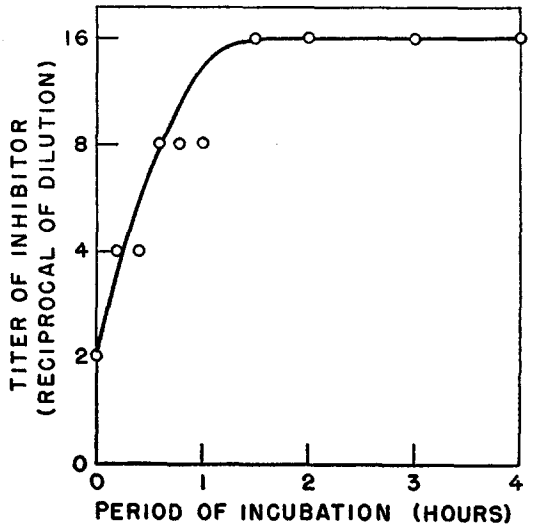

FIG. 2. Variation in hemagglutination-inhibition end-point as a function of the time that inhibitor and virus interact at 5°C. Inhibitor (0.3 ml.) and virus (0.2 ml.) were incubated for varying periods of time (abscissa) before addition of RBC (0.5 ml.). The ordinate represents the dilution (before addition of virus and RBC) of a 1 μg./ml. solution of inhibitor at the titration end-point.

When the interval between the 2 inocula was greater than 1 hour, separate virus controls were used. The results that were obtained are shown in Table V.

The findings recorded in Table V show that if virus is injected first, followed very shortly thereafter by inhibitor, there is no measurable inhibitory effect. If, however, the inoculation of inhibitor precedes that of virus, the inhibitory effect persists for at least 2 days and possibly longer. The effect of

the inhibitor is not restricted to the site of the inoculation, since as shown in Experiment 1 (Table V) the 2 inoculations were made in the same site, whereas in Experiment 2, the inoculations were made in opposite cerebral hemispheres. The results of both experiments are essentially the same. The above observations suggest that the inhibitor functions by combining with the virus and that this reaction is possible only when the virus is free; *i.e.*, before it comes in contact with its host cell.

To obtain more direct evidence that virus and inhibitor interact, use was made of an enzyme which specifically inactivates the inhibitor without affecting either the infectivity of the virus or the susceptibility of the host. The

TABLE V

Effect of Varying the Time and the Order of Inoculation of Virus and Inhibitor

Experiment No.	First inoculum	Time interval	Second inoculum	Infectivity-inhibition	
				$\frac{1}{T}$	Virus control, $\frac{1}{T}$
1	Virus	2 minutes	Inhibitor	0.21	0.25
	"	10 "	"	0.22	—
	Inhibitor	10 "	Virus	0.10	—
	"	1 hour	"	0.15	—
	"	1 day	"	0.17	0.32
	"	2 "	"	0.21	0.35
2	Inhibitor	1 day	Virus	0.13	0.31
	"	2 "	"	0.17	0.32
	"	1 week	"	0.23	0.29
	"	2 "	"	0.26	0.32

properties of this enzyme will be described below. If the virus and inhibitor interact to form a complex, centrifugation at high speed of a mixture of the two should yield a deposit which is devoid of viral activity. Treatment of the neutralized virus by the specific enzyme might be expected to restore viral infectivity.

A mixture consisting of 6.0 ml. of 10^{-3} dilution of virus and 2.0 ml. of an inhibitor solution (500 μg./ml.) was allowed to stand for 1 hour at 5°C. and then centrifuged for 2 hours at 140,000 *g*. The supernatant fluid was decanted as completely as possible. The sediment was resuspended in 8.0 ml. of saline and centrifuged for 2 hours at 140,000 *g*. After the supernatant fluid was discarded, the sediment was resuspended in 2.0 ml. of saline. One aliquot was treated with an equal volume of partially purified[2] enzyme for 1 hour at 37°C.; a second aliquot was treated with heat-inactivated enzyme. Both specimens were tested for infectivity in mice. The results are shown in Table VI.

[2] The authors wish to thank Mr. Irwin Schultz for assistance in the purification of the enzyme.

The data in Table VI show that the sediment from the virus-saline mixture had a higher titer (0.31) than the sediment from the virus-inhibitor mixture (0.20). Upon treatment of each with active enzyme, the titer of the latter increased about 100-fold (0.42), whereas the titer of the virus control remained the same (0.32). It is of interest that the titers of the specimens obtained from the virus-saline mixture were lower than the titer of the reactivated specimen. Since it had been determined that approximately 50 per cent of the total content of virus had been sedimented with each centrifugation of the virus control, the above difference in viral activity may indicate that the neutralized virus (*i.e.* virus-inhibitor complex) has a greater mass and therefore a higher sedimentation rate.

TABLE VI

Sedimentation from a Mixture of Virus and Inhibitor of Partially Neutralized Virus and Restoration of Viral Activity by an Inhibitor-Destroying Enzyme

Dilution	Values of $\frac{1}{T}$ obtained with			
	Sediment from virus-inhibitor mixture incubated 37°C./1 hr. with		Sediment from virus-saline mixture incubated 37°C./1 hr. with	
	Active enzyme	Inactive* enzyme	Active enzyme	Inactive* enzyme
Undiluted	0.42	0.20	0.32	0.31
1:5	0.32	0.11	0.26	0.27
1:25	0.29	—	0.22	0.19

* Enzyme inactivated by heating for 10 minutes at 100°C.

The Specificity of the Action of the GDVII Virus Inhibitor.—The GDVII virus inhibitor was tested for activity against several other neurotropic viruses. With Theiler FA virus the effect on the $1/T$ value was examined. In the case of TO(I), TO(B), and Lansing viruses the effect on the 50 per cent end-point (ID_{50}) was observed.

Dilutions of each virus were prepared and mixed with equal volumes of a 1 mg./ml. solution of purified inhibitor. The mixtures were incubated for 1 hour in the refrigerator. GDVII virus was included in the experiment as control. In this case, however, the concentration of the inhibitor was 0.01 mg./ml. The results are shown in Table VII.

As shown in Table VII, 5 μg./ml. of inhibitor reduced the titer of GDVII virus by about 2 log dilutions, whereas the infectivity of the other 4 viruses was unaffected by inhibitor concentrations of 500 μg./ml.

Effect of Virus Preparations on the GDVII Virus Inhibitor.—It has been shown (6, 9, 15–20) that certain viruses (*e.g.* influenza) are able to destroy the inhibitory activity of certain hemagglutination inhibitors. The possibility was therefore considered that those viruses which were not affected by the GDVII virus inhibitor might possess the power to destroy it.

To test this possibility, freshly harvested virus preparations (10 per cent extracts of infected CNS or feces) were mixed with an inhibitor solution and incubated for 3 hours at 37°C. The titer of the inhibitor was determined at the beginning and end of the incubation period after first heating an aliquot at 100°C. for 5 minutes. Heating at this temperature inactivates the virus but does not affect the titer of the inhibitor. The effect on hemagglutination-inhibition is shown in Table VIII.

TABLE VII

Failure of the GDVII Virus Inhibitor to Act against Other Neurotropic Viruses

Virus	Concentration of inhibitor	ID_{50} end-point		$\frac{1}{T}$ value	
		Virus plus inhibitor	Virus control	Virus plus inhibitor	Virus control
	μg./ml.				
TO(I)	500	$10^{-1.6}$	$10^{-1.6}$	—	—
TO(B)	500	$10^{-4.4}$	$10^{-4.5}$	—	—
Lansing	500	$10^{-2.9}$	$10^{-2.8}$	—	—
FA	500	—	—	0.21	0.21
GDVII	5	$10^{-4.7}$	$10^{-6.3}$	0.06	0.25

TABLE VIII

Effect on the Titer of the GDVII Virus Inhibitor of Several Viral Preparations

Virus	Hemagglutination-inhibition	
	Before incubation*	After incubation*
	HIU/ml.	*HIU/ml.*
TO(I)	6,400	800
TO(B)	6,400	6,400
Lansing	6,400	6,400
FA	6,400	6,400
Normal CNS	6,400	6,400
TO-free fecal extract	6,400	800

* The virus-inhibitor mixtures were incubated for 3 hours at 37°C.

As shown in Table VIII, there was no effect on the titer of the inhibitor by TO(B), Lansing, and FA viruses, nor by normal mouse CNS tissue. On the other hand, the TO(I) virus preparation, as well as a similar extract obtained from the feces of mice free of TO virus, caused a significant diminution in the titer. Since an inhibitor-destroying agent was present in the fecal extracts, no conclusion regarding inhibitor-destroying activity of TO(I) virus could be reached.

It was therefore decided to purify TO(I) virus by differential centrifugation and to follow the inhibitor-destroying activity during purification.

A crude 10 per cent extract of mouse feces was prepared and treated with 0.5 volume of ether overnight in the refrigerator. After removal of the ether, the aqueous extract was centrifuged for 1 hour at 32,000 *g*. The sediment was discarded and the supernatant fluid was

centrifuged for 2 hours at 140,000 *g*. The supernatant fluid was decanted and saved; the virus-containing pellet was suspended in $\frac{1}{50}$ of the original volume of water. Since the hemagglutination-inhibition method of measuring inhibitory activity has a relatively high experimental error, a more sensitive method (discussed below) of measuring inhibitor destruction was employed based on the amount of reducing sugars liberated from the mucopolysaccharide as a result of its destruction. Three of the above fractions were tested for inhibitor-destroying activity by incubating a sample of each with the inhibitor and measuring the reducing sugars before and after incubation.

TABLE IX

Inhibitor-Destroying Activity of Several Fractions Obtained during Purification of TO(I) Virus

Fraction	Enzymatic activity
	*total units**
32,000 *g* supernate	250
140,000 *g* supernate	169
140,000 *g* pellet	29

* 1 unit of enzyme liberates 1 μ M of reducing sugar (as glucose) from 1 mg. of inhibitor in a final volume of 0.5 ml. in 1 hour at 37°C.

TABLE X

Effect of a Crude Extract of Mouse Feces on the GDVII Virus Inhibitor

Time of incubation	Reducing sugars*	Hemagglutination-Inhibition	Infectivity-inhibition	
			$\frac{1}{T}$	Virus control, $\frac{1}{T}$
37°C./hrs.	*mg./ml.*	*HIU/ml.*		
0	0.65	64,000	0.04	0.28
1	1.01	6,400	—	
2	1.19	800	0.32	

* Expressed as glucose, determined by the method of Nelson (35).

The data in Table IX show that all three fractions possess inhibitor-destroying activity. After removal of the virus from the fecal extract by centrifugation, the bulk of the activity remains in the supernatant fluid. The virus-containing fraction, however, retains a low degree of activity. Whether this activity is in fact associated with the virus itself, or is derived from the supernatant fluid as a contaminant cannot be decided from the above experiment.

Enzymatic Inactivation of the GDVII Virus Inhibitor.—As shown above, the feces of mice contain an agent capable of destroying the biological activity of the inhibitor. Since the available evidence (30) indicates that the inhibitor is a mucopolysaccharide, an attempt was made to follow the inactivation of the inhibitor by measuring the change in reducing sugars as well as the reduction in titer. The results of a typical experiment in which the inhibitor was incubated with a crude fecal extract are shown in Table X.

It can be seen that there is a progressive increase in reducing sugars as the hemagglutination-inhibition and infectivity-inhibition titers decrease. When the inhibitor is incubated with a fecal extract which had been heated for 30 minutes at 56°C., the above noted changes do not occur. Incubation of the fecal extract without added inhibitor results in little or no change in reducing sugars. These results show that crude extracts of feces contain an enzyme, or enzymes, capable of degrading the mucopolysaccharide and reducing its inhibitory action.

An attempt was made to determine if the enzyme is produced by the microbial population of the intestinal tract of the mouse. When a fecal suspension was inoculated into neopeptone broth and incubated anaerobically for 24 hours at 37°C., inhibitor-destroying activity was present in the culture filtrate. After several subcultures of the mixed microbial population, enzymatic activity was still present. Isolation of a single bacterial species capable of synthesizing the enzyme has not been successful. Incubation has been carried out in the presence of inhibitor under aerobic and anaerobic conditions both in the presence and absence of CO_2.

Several known enzymes have been examined for inhibitor-destroying activity. Crystalline trypsin, ribonuclease, lysozyme, and spleen cathepsin[3] were found to be inactive. A filtrate from a culture of *Vibrio cholerae* was tested and found to be inert although it was very active in destroying the receptors on human RBC for influenza virus. A preparation of commercial pancreatin was found to be weakly active.

DISCUSSION

It has been shown that some viruses, *e.g.* influenza, are capable of reacting with various naturally occurring receptor substances. The reaction appears to be enzymatic in nature, the enzyme being associated with the virus particle. As with other enzyme reactions, there is first the formation of an enzyme-substrate complex followed by dissociation of the complex with alteration of the substrate. It has been shown that this course of events can be modified so that the formation of the complex occurs without subsequent dissociation. Hirst (36) accomplished this by treating the enzyme (virus) with periodate, whereas Burnet (37) treated the substrate (receptor substance) with periodate. In the latter case the substrate was modified sufficiently to lose its susceptibility to the action of the enzyme without losing its affinity for the enzyme. This modified substrate therefore behaves as an inhibitor, and Burnet has shown (37) that it is capable of inhibiting infection of chick embryos with the WSE strain of influenza virus.

In the present instance GDVII virus possesses no demonstrable enzyme activity against the intestinal inhibitor. This is perhaps the explanation for

[3] The authors wish to thank Dr. I. Krimsky for supplying the spleen cathepsin.

the rather unique property of the inhibitor of neutralizing viral infectivity as well as hemagglutination. Like other known inhibitors of viral hemagglutination, the GDVII virus inhibitor is a high molecular weight mucopolysaccharide.

The best source of inhibitor was found to be the small intestine of the adult mouse, which is also the probable site of multiplication of Theiler's TO virus. It was interesting to find that the same tissue obtained from infant mice which are free of TO virus, is a relatively poor source of inhibitor. The possibility that the inhibitor of GDVII virus is actually TO virus receptor substance was investigated without a satisfactory conclusion. Although TO virus, purified by high speed centrifugation, contained enzymatic activity against the mucopolysaccharide, the possibility that the virus was contaminated with a fecal carbohydrase could not be excluded. That there is an inhibitor-destroying factor independent of TO virus is clearly shown by the presence of enzymatic activity in the following preparations: (*a*) The supernatant fluid obtained after high speed centrifugation of TO virus; (*b*) Fecal extracts from mice free of TO virus; (*c*) Fecal extracts from human beings and rats which are free of TO virus.

With the aid of the inhibitor-destroying enzyme, evidence has been obtained which shows that the inhibition of GDVII virus is the result of the combination of virus and inhibitor. The possibility should be considered that a similar reaction may occur in the case of "masked" or "latent viruses." Reactivation of a "masked" virus by an enzyme has been demonstrated with PVM (38).

The inhibitory effect of extracts of various tissues from mice on GDVII virus was studied by Fastier (39). He found that extracts of various organs inhibited hemagglutination but not infectivity; however, no data on intestinal tissue were included.

It is of interest that Theiler's FA virus is entirely resistant to the action of the mucopolysaccharide inhibitor in spite of the fact that it is closely related to GDVII virus on the basis of complement-fixation (40) and hemagglutination-inhibition (32) studies. Other differences in biological behavior between these two strains have been observed. For example, FA virus fails to grow in tissue culture (41) under conditions which support multiplication of GDVII virus (42). Also, FA virus inhibits carbohydrate metabolism in mouse brain, whereas GDVII does not (43). Finally, GDVII but not FA agglutinates human erythrocytes (32).

SUMMARY

A mucopolysaccharide has been obtained from intestinal tissue of adult mice which inhibits both infectivity and hemagglutination of Theiler's GDVII strain of encephalomyelitis virus of mice. The inhibitor is inactive against the FA and TO strains of Theiler's virus and against the Lansing strain of polio-

myelitis virus. In the adult mouse, large amounts of the inhibitor are found only in the small intestine. The small intestine of infant mice, however, contains a considerably smaller amount of inhibitor. Inhibition, both *in vivo* and *in vitro*, appears to be the result of an interaction between virus and inhibitor. The intestines of man, monkey, rabbit, rat, cotton rat, hamster, sheep, cow, and pig contain relatively little inhibitor whereas guinea pig intestine contains as much as adult mouse intestine. An enzyme was found in the feces of mice, and several other animals, which is capable of destroying the inhibitory activity of the mucopolysaccharide with the liberation of reducing sugars.

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