Initial Stages of Mustard Action Upon Influenzal Toxicity and Infectivity.* (20305)

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A previous paper(1) has shown that exposure of influenza A (PR8) virus to appropriate concentrations of sulfur mustard or various chloroethylamine derivatives resulted in considerable inactivation of the toxic and infective principles of virus. Analysis of the time relationship in inactivation of these two viral attributes demonstrated the rapidity of destruction of both viral properties and a seemingly greater inactivation of viral infectivity as opposed to viral toxicity. In this earlier investigation it had not been possible to study the initial phases of mustard action upon influenzal toxicity because of the complicating factor of chemical intoxication following intracerebral inoculation of materials containing unreacted mustard or its intermediates. The possibility of either an equivalent rate of destruction of viral toxicity and infectivity or a selective action of mustard upon one or the other of the two viral characteristics in the initial stages of mustard-virus interaction was, therefore, not ascertained. The present report describes the use of sodium thiosulfate as a means of circumventing chemical intoxication and the nature of mustard action upon influenzal toxicity and infectivity in the early periods following exposure of influenza A (PR8) virus to one of the chloroethylamine derivatives.

Materials and methods. Virus. An egg-adapted strain of influenza A (PR8) virus with a continuous history of allantoic passage in embryonated eggs since isolation was used in these studies. Virus was prepared by suballantoic inoculation of 10- or 11-day embryonated eggs with 0.05 ml of a 10^{-6} or 10^{-7} dilution of virus in beef-heart infusion broth. After incubation of infected eggs at 36°C for 40 to 48 hours, the eggs were chilled overnight in the refrigerator and the allantoic fluids harvested. The pooled fluids were stored in the refrigerator and centrifuged at 2400 rpm for 20 minutes before use to remove coarse particles which had accumulated upon standing. The toxicity and infectivity of the preparation was determined each week, and whenever any significant loss of activity in either of the viral properties was noted, a new suspension of infected allantoic fluid was prepared. *Mustard.* The chloroethylamine derivative used throughout these investigations was cyclohexyl [bis(β-chloroethyl)] amine hydrochloride, kindly supplied by Dr. H. B. Woodruff of Merck & Co., Inc. A 0.05 M solution of chemical was prepared by dissolving weighed quantities of the chemical in cold, triple-distilled water of pH 2.6. This was done to eliminate variations in ionic content of water and to repress cyclization of mustard until ready for addition to virus. *Exposure of virus to mustard.* An appropriate amount of the 0.05 M solution of mustard was added to a given volume of chilled viral suspension to yield the desired concentration of chemical. The mustard-virus suspension was thoroughly mixed and placed in an ice-water bath at 4°C. Samples were removed at selected intervals for determination of viral toxicity and infectivity. Only freshly prepared solutions of mustard were employed in these tests and they were added to virus as soon as complete dissolution of chemical had occurred. *Concentration of mustard-treated virus.* Suspensions of mustard-treated virus were adsorbed with 5% chicken red cells in the cold. After 45 minutes in an ice-water bath, the supernatant fluid was removed as completely as possible, and the agglutinated cells washed with 0.1 M phosphate buffer. The washed cells were allowed to resettle in the cold. Following removal of the supernatant fluid, a volume of 0.1 M phosphate buffer of pH 7.4, equivalent to 1/10th of the original volume of mustard-virus suspension, was added to the agglutinated cells. Elution of adsorbed virus was then carried out in a 37°C water bath for

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4 hours. At the end of this period the suspension was centrifuged at 2400 rpm for 20 minutes and the supernatant fluid removed and tested for viral toxicity, infectivity and hemagglutinins. *Test of viral toxicity.* At the time selected for testing of mustard-treated virus for toxicity, 0.04 ml of a 5 M aqueous solution of sodium thiosulfate was mixed with 1.96 ml of mustard-virus suspension. This represented the undiluted mustard-virus preparation from which serial 2-fold dilutions of virus were made in 0.1 M aqueous sodium thiosulfate for determination of residual toxicity of virus. The test for viral toxicity was made by inoculating 0.03 ml of virus into the right cerebral hemisphere of mice under light ether anesthesia. The animals used in the tests were Swiss mice (Webster strain), 12 to 15 g weight and of random sex. Mice which were obviously traumatized by the intracerebral injections were discarded. Deaths or convulsions (upon spinning of the animals) 24 hours after injection were used as criteria for virus-induced toxic reactions. All mice were observed daily for a period of 7 days. The dilution of virus causing toxic reactions in 50% of the experimental animals was calculated by the method of Reed and Muench(2). *Toxicity of viral progeny derived from mustard-treated virus.* The toxicity of viral progeny derived from mustard-treated virus after one allantoic passage in 10- or 11-day embryonated eggs was determined in a similar manner. The propagation of mustard-treated virus was carried out in the manner described below for the infectivity test. Only the fluids from eggs infected with a single infective dose of mustard-treated virus were tested in this respect. *Infectivity test.* Mustard-treated virus was diluted serially in 10-fold steps in beef-heart infusion broth. The starting point of the dilution series was the undiluted sample of mustard-virus suspension containing 0.1 M sodium thiosulfate as prepared for determination of viral toxicity. The dilutions of virus were inoculated suballantoically in 0.05 ml amounts into groups of 4 to 6 embryonated eggs of 10- or 11-days incubation. After 40 to 48 hours at 36°C, the individual allantoic fluids were tested for presence of viral hemagglutinins by mixing 0.1 ml of fluid with 0.25 ml of 1% chicken erythrocytes. The test was read by the pattern method(3) after 30 minutes at room temperature. The ID_{50} per 0.05 ml of suspension was calculated by the method of Reed and Muench(2). *Hemagglutination test.* Titration of viral hemagglutinins in the mustard-virus suspensions concentrated by the method of red cell adsorption and elution was made by using serial 2-fold dilutions of virus in saline and mixing 0.5 ml of viral dilution with 0.5 ml of 1% chicken red cells. The test was read after 30 minutes at room temperature by the pattern method(3). The end point was taken as the last tube showing complete agglutination, and the titer expressed as the reciprocal of the dilution of virus in this tube.

Results. Effect of sodium thiosulfate upon viral toxicity and infectivity. Studies of the action of aqueous solutions of sodium thiosulfate upon the toxic and infective components of influenza A (PR8) virus (results not shown) revealed that concentrations of 0.2 M thiosulfate or less caused no inactivation of either of the viral attributes. The dilution of virus causing toxic reactions in 50% of experimental animals and the number of egg-infective doses remaining after contact of virus with 0.2 M sodium thiosulfate for one hour at room temperature (24°C) were identical with the values observed for untreated virus. The action of higher concentrations of sodium thiosulfate upon virus was not investigated.

Toxicity of sodium thiosulfate for intracerebrally injected mice. The tolerance of mice for intracerebral inoculation of 0.03 ml of various concentrations of aqueous solutions of sodium thiosulfate is shown in Table I. The data of this table represent the combined results of two separate experiments. It is evident that concentrations of 0.075 M or less of sodium thiosulfate were well tolerated. No fatalities or ill effects were observed in any of the animals during an observation period of 7 days. Intracerebral injections of 0.1 M sodium thiosulfate proved toxic to slightly less than 50% of experimental animals. Still higher concentrations of chemical (0.25 to 0.5 M) were 100% lethal.

Reduction of mustard toxicity by sodium

TABLE I. Toxicity of Sodium Thiosulfate for Intracerebrally Injected Mice.

	(M) sodium thiosulfate inoculated						
	.5	.25	.125	.1	.075	.065	.05
No. dead / No. inoc.	16/16	16/16	8/16	7/16	0/16	0/16	0/16

TABLE II. Reduction of Mustard Toxicity by Sodium Thiosulfate.

(M) mustard in allantoic fluid	(M) thiosulfate in allantoic fluid	Intracerebral toxicity of sample: Min. after dissolution of mustard 0	5	15	30
5×10^{-4}	0	6/6*	6/6	6/6	4/6
"	.075	0/6	0/6	0/6	0/6
"	.1	0/6	—	—	0/6
0	.075	0/6			
"	.1	2/6			

* Numerator designates No. of animals dead. Denominator indicates total No. of animals in test.
— indicates sample not tested.

TABLE III. Initial Stages of Mustard Action upon Influenzal Toxicity and Infectivity.

(M) mustard added to virus	Time (min.) of sampling	Toxicity of sample: Undil.	1:2	1:4	1:8	Dilution causing 50% toxic reaction of sample	Log ID_{50}
5×10^{-4}	Immediate	3/6*	1/6	0/6	0/6	1:1.1	7.5
"	5	1/6	0/6	—	—	<1:1	7.0
"	10	1/6	0/6	—	—	"	6.3
"	20	0/6	—	—	—	"	5.5
"	30	0/6	—	—	—	"	5.2
0		6/6	5/6	4/6	0/6	1:4.3	7.5

* Numerator refers to No. of animals showing toxic symptoms. Denominator refers to total No. of animals in test.
— indicates sample not tested.

thiosulfate. It may be seen from the results of Table II that intracerebral inoculation of 0.03 ml of 5×10^{-4} M mustard in normal allantoic fluid proved highly toxic to mice. All of the animals succumbed within a few hours after inoculation. The toxicity of the preparation was almost as great after 30 minutes at room temperature as in the first few minutes. Addition of 0.075 M sodium thiosulfate to the mustard-containing allantoic fluid immediately eliminated the lethality of the preparation, for none of the experimental animals given mustard-thiosulfate allantoic fluid died during the observation period of 7 days. It is of interest that admixture of 0.1 M sodium thiosulfate with mustard-containing allantoic fluid not only rendered the latter entirely innocuous to mice, but resulted also in reduction of the toxicity of sodium thiosulfate itself. The nullifying action of sodium thiosulfate upon mustard toxicity thus provided a means for studying the toxicity of virus during the early stages of mustard-virus interaction without the complicating factor of chemical intoxication.

Nature of mustard action upon influenzal toxicity and infectivity in the early stages of mustard-virus interaction. The data of the experiment shown in Table III are representative of the results obtained in several identical experiments. There seemed to be an extremely rapid destruction of the toxic component(s) of virus following addition of mustard to virus. As compared with the toxic content of untreated virus, and reflected in the dilution of virus causing toxic reactions in 50% of animals, an approximate 4-fold drop in viral toxicity may be observed for mustard-treated virus within the first few moments of reaction. In the succeeding intervals (5 to 30 minutes) intracerebral injection of mice with mustard-virus suspension provoked only an occasional

TABLE IV. Toxicity of Mustard-Treated Virus after Concentration by Red Cell Adsorption and Elution.

Sample*	(M) mustard added to virus	HA† titer	Toxicity of sample: Undil.	1:2	1:4	1:8	Dilution causing 50% toxic reaction
MV	5×10^{-4}	512	0/6‡	—	—	—	<1:1
MVC	"	4096	0/6	0/6	0/6	—	"
MV	1.25×10^{-4}	512	6/6	3/6	0/6	—	1:2
MVC	"	4096	5/6	5/6	3/6	1/6	1:3.6
Virus	0	512	6/6	6/6	5/6	2/6	1:6.1

* MV refers to mustard-treated virus after 30 min. at 4°C. MVC refers to same preparation after concentration.

† Hemagglutinin titer expressed as reciprocal of dilution of virus in last tube showing complete agglutination.

‡ No. of animals showing toxic symptoms/No. of mice in test.

toxic response. In contrast to the findings recorded for the action of mustard upon viral toxicity, there was no immediately observable inactivation of the infective component(s) of influenza virus by mustard. Instead, the course of inactivation of viral infectivity appeared to be a gradual and orderly process, as evidenced by the progressive reduction in level of egg-infective virus at each of the succeeding intervals.

Toxicity of mustard-treated virus after concentration by red cell adsorption and elution. The results shown in Table III have established the rapidity of destruction of influenzal toxicity by 5 x 10^{-4} M mustard, but carry no inferences concerning the completeness or permanency of this loss. These two aspects were investigated by a) analysis of the toxicity of mustard-treated virus after concentration of virus by the method of red cell adsorption and elution, and b) study of viral progeny obtained from eggs infected with one ID_{50} of mustard-treated virus. The results are presented in this and the following section.

Table IV indicates that destruction of influenzal toxicity by 5 x 10^{-4} M mustard was apparently complete after 30 minutes at 4°C. This was suggested by the absence of toxic reactions in mice given intracerebral injections of mustard-treated virus which had been concentrated 8-fold (as reflected in an increased hemagglutinating titer). In comparison, it may be seen that exposure of virus to 1.25 x 10^{-4} M mustard for the same period of time resulted in only an approximate 3-fold drop in viral toxicity. An 8-fold concentration of this particular preparation produced almost a doubling of the residual viral toxicity. That the increased toxicity of the latter was not ascribable to chemical intoxication was indicated by the absence of toxicity of the concentrate prepared from the viral suspension treated with 5 x 10^{-4} M mustard.

Toxicity of viral progeny derived from mustard-treated virus. Table V shows the toxic behavior of a few selected examples of viral progeny derived from mustard-treated virus. It is readily evident that viral progeny of high and low toxicity may be encountered and that no definite relationship between hemagglutinin titer and level of viral toxicity can be detected. This particular behavior of virus derived from cultivation of single infective units of mustard-treated virus in embryonated eggs closely simulated the toxic pattern of untreated virus passaged in an identical manner (results for latter not shown). Examination of over twenty such single viral isolates revealed no instances of non-toxic viral suspensions.

Discussion. Previous investigations of the action of mustards upon viruses have been confined largely to problems of pragmatic significance, and utilization of these chemi-

TABLE V. Toxicity of Viral Progeny Derived from Mustard-Treated Virus.

Sample	HA titer	Toxicity of sample: Undil.	1:2	1:4	Dilution causing 50% toxic reaction
1	320	6/6*	6/6	4/6	>1:4
2	"	"	3/6	0/6	1:2
3	"	5/6	0/6	"	1:1.3

* No. of animals showing toxic symptoms/No. of mice in test.

cals as selective agents for inactivation of functional groups of viruses has not been explored in general. An earlier report(1) on the action of mustards upon influenzal toxicity and infectivity had demonstrated a concurrent and rapid inactivation of both viral attributes and a seeming lack of preferential action of mustards upon virus. These findings, however, represented essentially the end results of mustard action, and the possibility of an early preferential action of mustard upon virus was not excluded. Obvious hindrances to an analysis of the initial stages of mustard action upon influenzal toxicity and infectivity were extreme rapidity of mustard action and residual chemical toxicity. As described in another paper(4), deceleration of mustard inactivation of virus was attained by a reduction in temperature of the reacting system. The present report demonstrated the detoxification of residual mustard toxicity by sodium thiosulfate. Utilization of these two observations in studying the early phases of mustard-virus interaction has shown a seemingly rapid destruction of viral toxicity and a more gradual but progressive inactivation of viral infectivity by cyclo mustard. The results were suggestive of an early preferential action of mustard upon influenza A (PR8) virus. While the possibility of a continuing action of mustard upon virus following inoculation of mustard-virus suspension into the central nervous system cannot be eliminated, it does not seem too likely in view of the dilution factor and the many reactive constituents in brain tissue.

Various studies(5-8) have shown that mustards increase mutation rates in *Drosophila*, bacteria and fungi. It might be assumed that viruses may likewise be subject to the mutational effect of these chemicals. The findings reported herein showed that, despite the apparently complete destruction of viral toxicity as evidenced by inability to restore this property after concentration of mustard-treated virus, no lasting effects were demonstrable. This was indicated by the occurrence of toxic manifestations in animals receiving intracerebral injections of viral progeny derived from mustard-treated virus.

Summary. 1. Sodium thiosulfate in concentrations of 0.2 M or less produced no observable effects upon viral toxicity and infectivity. 2. Intracerebral inoculation of 0.075 M or less of sodium thiosulfate was well tolerated by mice. 3. Admixture of 0.1 M sodium thiosulfate with 5×10^{-4} M cyclo mustard resulted in detoxification of the latter. 4. A possibly greater rate of destruction of viral toxicity by mustard, as opposed to viral infectivity, seemed to occur in the early stages of mustard-virus interaction. 5. Inactivation of viral toxicity by mustard proved complete, but the loss was not demonstrable in the viral progeny derived from eggs infected with one ID_{50} of mustard-treated virus.

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