atypical replication of the influenza virus will occur in tissues other than the brain of the mouse, or in any of the tissues of the rat, which is not susceptible to influenza infection.

Summary. Aureomycin and terramycin, administered subcutaneously and orally, did not increase the resistance of mice or rats to the acute toxic action of influenza A virus.

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Mustard Inactivated Rabies Vaccine. (18252)

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Our preliminary paper(1) on the production of vaccines by treating virus suspensions with mustard was based in part on studies in this laboratory on the effect of mustard on proteins. These studies(2), which were published subsequently to our paper, showed among other things that relatively small amounts of mustard inactivated enzymes and that mustard reacted with the carboxyl groups of protein molecules but not with the free amino groups. After our preliminary paper, one of us(3) studied the action of mustard on animal, plant, and bacterial viruses, as well as on bacteria, yeasts, and a pneumococcus-transforming agent, and found that viruses and cells were inactivated faster than enzymes and that viruses containing desoxyribose nucleic acid were inactivated faster than those containing ribosenucleic acid. Mustard gas seems an ideal inactivating agent because its action is complete within a relatively short time and in our experiments the end products were not toxic. Mustard is bactericidal; and if the contamination of the starting material is not too great, sterile vaccines are secured. It is in fact much more like ultraviolet light in its action than like most other chemical agents. When tested for antigenicity it is found that inactivated rabies and equine encephalomyelitis viruses immunize when diluted 10^{-3} or higher, hog cholera and Newcastle viruses immunize when undiluted, while very preliminary experiments with pseudorabies and Baker's cat viruses have failed to show antigenicity.

In the present paper some experiments made with inactivated rabies virus are reported, since the vaccine promises to be of practical importance and since the findings may be useful as a guide for the preparation of other vaccines.

Materials and methods. The fixed viruses used were a strain obtained from the Bureau of Laboratories of the Department of Health of the City of New York, through the kindness of Dr. Jules Freund, the Pitman-Moore strain 980 from Dr. R. B. Gochenour, and a strain adapted to mice from Dr. Harald Johnson. The street virus used for challenging the guinea pigs also came from Dr. Johnson. Rabbits, guinea pigs, and mice were inoculated intracerebrally and the brains removed when the animals were completely paralyzed. Small amounts of brain material were suspended in water, using a glass grinder; larger amounts were suspended in water in a Waring blender. Water was used rather than saline as the reaction is retarded by halid ions.

Test of immunity. The least amount of antigen injected into the peritoneal cavity that would immunize guinea pigs weighing 250-300 g against street virus was determined. This test has been described in some detail(1).

Mustards. Sulfur mustard (*bis* (β-chloroethyl) sulfide) was prepared in this laboratory

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1. TenBroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 271.

2. Herriott, R. M., Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1946, v30, 185.

3. Herriott, R. M., *J. Gen. Physiol.*, 1948, v32, 321.

by the method of Bent(4) followed by fractional crystallization. The final product melted at 14.4°C. The 3 nitrogen mustards were obtained from Edgewood Arsenal.

Inactivation with sulfur mustard. A saturated solution was obtained by adding 0.01 ml of mustard to each 15 ml of sterile distilled water taken immediately from the refrigerator at an approximate temperature of 3°–10°C. This gives a concentration of about 5×10^{-3} molar. The distilled water is contained in a glass-stoppered cylinder, and in introducing the mustard the pipette is kept from the sides of the cylinder and the tip is placed below the surface of the water so that the mustard sinks. The cylinder is immediately closed and shaken vigorously for approximately 30 seconds. An equal part of the saturated solution is added to a 20% brain suspension warmed to 25°C, giving a 10% brain suspension half saturated with mustard. If lower concentrations are needed, an appropriate amount of distilled water is added to the brain suspension, then the proper amount of saturated mustard, so that the final concentration of brain is always 10%. The reaction of the brain suspension is adjusted to pH 7.3 before mixing with the mustard solution. In its reaction with water mustard produces acid and the pH of the mixture is determined for the first ½ hour by the drop method, and normal sodium bicarbonate sterilized by filtration is added dropwise to keep the pH at approximately 7.3. After 2 hours at 25°C the preparations are placed in the refrigerator.

Precautions to prevent burns with mustard. The mustard is kept in a glass-stoppered cylinder. Immediately after use the contaminated pipettes are placed in alcohol and then washed in warm water for 15 minutes before they are transferred to cleaning fluid. The cylinders used are treated in the same way. In the more than 3 years work there has not been a single burn.

Tests for inactivity of the virus. The routine test for inactivation has been the intracerebral injection of 0.1 ml of the 10% treated material into each of 3 guinea pigs the day after the preparation of the vaccine. These animals are observed over a period of a month. Further tests for the presence of active virus will be described below.

TABLE I. Protection by Inactivated Vaccines.

Source of brain suspension	No. tested	Protection of 50% of the guinea pigs by 1 ml of a concentration of		
		.008% (excellent)	.04% (good)	.2% (poor)
Guinea pig	6	1	2	3
Mouse	7	5	1	1
Rabbit	20	3	11	6

Results. With rabies and equine encephalomyelitis viruses it has been repeatedly shown that the best vaccines are those produced by the least amount of mustard that will inactivate. When the starting material is fixed virus in rabbit brain, ½ saturation always inactivates a 10% suspension, whereas 1/4 saturation will rarely do so. The ½ saturated material is a much better antigen than that produced by complete saturation. When 5% mouse brain is used a 1/4 saturated preparation is inactive and is a better antigen than that produced by ½ saturation. Thirty-three preparations have been treated with mustard and their antigenicity determined. The results are given in Table I and show that while all of them immunize, some preparations are better than others. Guinea pig brain although it has a high virus content does not make a good antigen when tested by the method used. Mouse brain is almost invariably good, and rabbit brain varies considerably. The best rabbit brain preparations we have had were from suspensions made in other laboratories where large numbers of rabbits were inoculated and the brains combined. In our laboratory, where small numbers of rabbit brains were combined and treated with mustard, most of the resulting antigens could be classed as good.

Comparison of mustard inactivated vaccines with those inactivated with phenol or irradiated light, and with untreated fixed virus. In order to compare the mustard preparations with those inactivated by other means we secured the help of 2 outside laboratories that were producing vaccines by the

4. Bent, H. E., *Science,* 1947, v106, 374.

TABLE II. Comparison of Antigenicity of Rabbit Brain Fixed Virus Inactivated by Phenol and Mustard.

Date of preparation	50% end point in mice before inactivation .03 × 10⁻	Age of vaccine when tested, days	Inactivated by	% suspension inj. intraperitoneally (1 ml) and results of challenge with street virus intramuse. 3 wk later*			Controls
				0.5	0.1	0.02	
5/8/47	7.1	99	Phenol	7/7†	5/5	5/5	7/7
			Mustard	0/6	1/5	1/5	
				0.5	0.2	0.05	
6/17	6.8	22	Phenol	6/6	4/6	6/6	6/6
			Mustard	0/6	3/6	5/6	
				0.2	0.04	0.008	
9/23	6.67	52	Phenol	0/5	3/5	5/5	5/5
			Mustard	1/5	2/5	3/5	
				0.2	0.04		
6/8/48	7.5	112	Phenol	2/5	5/6		5/6
			Mustard	1/6	2/6		
				0.2	0.04		
9/28	7.8	30	Phenol	4/6	3/6		5/6
			Mustard	0/6	5/6		

* In Tables III, V, and VI, this heading has been shortened to "Results of challenge."
† In all tables, numerator indicates the no. of animals that died; the denominator, no. of animals inoculated.

TABLE III. Comparison of Mustard Inactivated and Irradiated Rabbit Brain Fixed Virus.

Date of preparation	50% end point in mice before inactivation .03 × 10⁻	Age of vaccine when tested, days	Inactivated by	Results of challenge*		Controls
				.2	.04	
3/5/48	6.5	10	Irradiated	1/5	4/5	4/5
			Mustard	1/5	4/5	
5/3	6.0	10	Irradiated	2/6	2/6	5/6
			Mustard	1/6	5/6	
5/21	6.0	11	Irradiated	2/6	2/6	6/6
			Mustard	0/6	3/6	
1/30/50	6.0	10	Irradiated	2/8	6/8	8/8
			Mustard	2/8	3/8	
4/3	6.17	51	Irradiated	3/8	6/8	7/8
			Mustard:			
			¼ saturated	2/8	7/8	
			½ "	1/8	6/8	

* See Table II.

recognized methods. They provided brain suspension for inactivation by mustard in our laboratory and permitted us to compare it with their own product made on the same day.

Comparison with phenol vaccine. With the assistance of Dr. Jules Freund, 5 different rabbit brain suspensions were secured from the Bureau of Laboratories of the Department of Health of the City of New York, treated with mustard, and then compared with the phenol inactivated vaccines made in the New York City laboratories on the same day. The results are given in Table II and show that the mustard inactivated preparations are invariably better than those inactivated by phenol.

Comparison with irradiated vaccine. Similar comparisons were made between irradiated and mustard inactivated preparations with the help of Drs. Hilleman and Nigg of E. R. Squibb & Sons. The results of the 5 tests, given in Table III, show that irradiated and mustard treated antigens have about the same antigenicity when tested on guinea pigs. It should be added that in the

TABLE IV. Comparison of Active Fixed Virus and the Same Virus Inactivated with Mustard as Immunizing Agents.

Virus strain	Brain suspension of	Vaccination with virus	Intracerebral titer in mice after freezing .03 × 10–	% brain in virus or vaccine and results of challenge with street virus intramusc. 3 wk after vaccination				Controls
				.1	.01	.001	.0001	
Georgia	Mouse	Active	5.76	—	0/5*	4/8	8/8	6/8
		Mustard inactivated		0/7	1/8	—	—	
Gochenour	Rabbit	Active	5.4	—	6/8	8/8		6/8
		Irradiated		3/8	—	—		
		Mustard inactivated		5/8	—	—		
				.2	.04	.01		
Gochenour	Rabbit	Active	5.6	—	4/7†	6/8		7/8
		Mustard inactivated:						
		¼ saturated		2/8	7/8			
		½ saturated		1/8	6/8			

* 3 of 8 guinea pigs died of fixed virus before the challenge.
† 1 of 8 guinea pigs died of fixed virus.

Squibb laboratories, Habel potency tests were done on these vaccines and all of the irradiated material passed the test while only one of the 5 mustard vaccines passed.

Comparison with untreated fixed virus. In 3 experiments, the results of which are given in Table IV, the immunizing action of active fixed virus is compared with that of the same virus inactivated with mustard. The active virus was placed in ampules and frozen with dry ice in alcohol, then kept under dry ice until the test injections were made. The results show that fixed virus has about 5 to 10 times the immunizing action of mustard inactivated material. It should, however, be noted that the 0.01% of fixed virus which immunized solidly was near the minimal infectious dose as some of the guinea pigs died from fixed virus infection before the challenge.

Other types of mustards. Preliminary tests have shown that the nitrogen and methyl sulfur mustards will inactivate fixed virus without destroying all antigenicity. Careful comparative tests between the vaccines produced by these and ordinary mustard have not been made. For the preparation of large amounts of vaccine the nitrogen mustards have the advantage that they can be weighed and that they are readily soluble in water. Sulfur mustard has to be shaken vigorously to saturate water and this is difficult with amounts of over 1 liter. However, it can be dissolved in alcohol or one of the "cellosolves" and then added to the water.

Toxicity of the vaccine. When a vaccine is prepared with a substance such as mustard, the first question that arises is whether the resulting preparation is toxic. It is well known that sulfur mustard disintegrates in water to yield a small quantity of a toxic material(5). However, no evidence of toxicity or irritation has been noted following intracerebral or intracutaneous injection of 0.1 ml undiluted vaccine into guinea pigs, or after intraperitoneal or subcutaneous injection of 1 ml of the same; after intraperitoneal or subcutaneous injection of 5 ml undiluted vaccine into dogs; or after intraperitoneal or intracerebral injection of 0.1 ml vaccine diluted to 1% brain suspension into mice. No tests have been made on man.

Drying of the vaccine. When frozen and dried, there is considerable loss of antigenicity, as shown in Table V. However, once dried, the antigenicity, as would be expected, remains unchanged for a long period. In 2 such samples tested there was no change over a period of 2 years in samples kept in the refrigerator.

Stability of the vaccine. As shown in Table VI, the vaccine is quite stable at refrigerator temperatures. Two of the samples tested more than a year after preparation

5. Stein, W. H., Moore, S., and Bergmann, M., *J. Org. Chem.*, 1946, v11, 664.

TABLE V. Effect of Freezing and Drying on Vaccines.

Vaccine	Results of challenge* .2	.04	.008	Controls
	A.			
Original	0/5	3/5	4/5	
Frozen and dried	3/5	5/5	4/5	
	B.			5/5
Original	0/5	0/5	2/5	
Frozen and dried	0/5	3/5	3/5	

* See Table II.

TABLE VI. Keeping Properties.

Vaccine	Interval between preparation and test	Results of challenge* .1	.04	Controls
Guinea pig brain	110 days	1/5	1/5	7/7
	250 "	0/5	4/5	5/5
		.2	.04	
Rabbit	99 days	1/5	1/5	7/7
	245 "	0/5	4/5	5/5
"	15 days	0/6	0/6	6/6
	1 yr. 7 mo.	0/6	0/6	4/5
"	11 days	0/6	4/6	6/6
	91 "	0/6	2/6	6/6
	1 yr. 3 mo.	0/6	1/6	4/5

* See Table II.

were almost as active as at the time of their first test. The other 2 samples showed some loss of antigenicity but they were still good immunizing agents.

Tests for active virus. As noted above, some of the vaccine preparations are extremely active, immunizing when diluted 1:1000 or more, and the question arises: Is active virus present which can be demonstrated by tests other than the usual one of intracerebral inoculation of undiluted vaccine? It has been impossible to show that active virus is present by inoculating guinea pigs intracerebrally with a good vaccine, removing the brains from these animals at 2, 4, and 6 days, and testing for virus by intracerebral injection into normal guinea pigs. Furthermore, blind passage from such animals in guinea pigs has failed to demonstrate virus. No evidence for a mutant that was avirulent but antigenic was obtained. No active virus could be detected from a vaccine that had a high immunizing property and that was diluted in distilled water 1:10, 1:100, and 1:1000, and after standing for 1 hour in the refrigerator was injected intracerebrally into mice. Andrewes and Elford (6) reported that when ectromelia irradiated vaccine is mixed with active virus and the mixture injected intraperitoneally, no infection results. In other words, the active virus is masked or interfered with. They suggest that the activity of some vaccines may be due to masked virus. Their experiment was repeated, using rabbit brain virus inactivated with mustard, as well as the same brain inactivated by ultraviolet light. The vaccines were diluted so that they contained 5% brain suspension, and to 0.9 ml of this vaccine was added 0.1 ml of known dilutions of fixed virus in milk. Mice were inoculated intracerebrally with 0.03 ml of these mixtures. Ten mice were inoculated with each, but the brain suspension itself was quite toxic and many of the mice died immediately. The results of the experiment are given in Table VII and it is clear that there was no masking effect by either the mustard or light inactivated vaccine.

Neutralizing antibodies. The activity of vaccines has been judged by their ability to protect guinea pigs against street virus, but some tests have been made for the presence of neutralizing antibodies. When low dilutions of these vaccines are injected, neutralizing antibodies have been detected some 3 weeks later. They have not been detected when higher dilutions, such as 1:250 or 1:1000, have been used, yet such dilutions have protected the guinea pigs. The test for immunity is more sensitive than the test for neutralizing antibodies 3 weeks after vaccination.

Discussion. There are a great variety of mustards and some or all of them may be used to produce vaccines. It is possible that one or more of these are better than sulfur mustard, but more is known about the last and it is the one to be preferred at present. It seems to us remarkable that every virus so far tested has been inactivated with mustard and with those examined in detail there has been retention of some immunizing

6. Andrewes, C. H., and Elford, W. J., *Brit. J. Exp. Path.*, 1947, v28, 278.

TABLE VII. Summary of Experiment to Determine whether Vaccine Would Act as an Interfering Agent when Mixed with Active Virus.

Vaccine Rabbit brain	Dilution of virus in 5% vaccine					Vaccine controls
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Inactivated with mustard	6/6	8/8	8/9	2/8		0/7
Irradiated, Squibb	9/9	10/10	8/9	5/6		0/8
Control. Virus diluted in milk		10/10	5/10	1/10	0/10	

property. Some of these antigens have been excellent while others will not immunize if diluted. However, the method should be useful in producing antigens to some of the viruses that produce disease in man. It also seems noteworthy that highly active vaccines have been produced against rabies from starting material of not especially high titer. It may be that the titer does not necessarily measure the amount of virus protein. If active brain material is suspended in strong buffer at pH 8 and titered immediately and again after 1 or 2 hours, it is found that the second titer is appreciably higher than the first, suggesting that the virus is aggregated and that in the buffer there is some dispersion. It is also possible that part of the antigenic material is not active virus but a precursor.

Although a relatively small amount of mustard is used to inactivate viruses and from isotopic studies it was found that a large proportion of the mustard reacts with the water, a rough calculation indicates that there must be several thousand mustard particles reacting with each virus particle. However, this is a reflection of the large particle size of the virus for on a percentage basis the amount bound is small. Certain reactions of mustard with simple proteins are understood but no comparable information is available for the reactions with nucleic acids or nucleoproteins.

Summary. The above work shows that mustard gas added to brain suspensions from animals infected with fixed virus gives a vaccine which by the method of testing used is better than that produced when the same brains are inactivated with phenol, about equal to that when the inactivation is by ultraviolet light, and almost as good as active fixed virus. The vaccine is very stable at refrigerator temperatures, and the method of preparation is simple and requires no unusual laboratory equipment.

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Prevention of Street Virus Infection by Vaccine.* (18253)

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Webster(1), in a review of the literature up to 1936, concluded that there is no good experimental evidence that rabies vaccine given after the injection of street virus will prevent the development of the disease. Since that date we have not found published experiments on this phase of rabies immunization. In the preceding paper(2) the evaluation of rabies vaccines was made by challenging guinea pigs 3 weeks after the intraperitoneal injection of known dilutions of

* The painstaking and accurate technical assistance of Mrs. Laura Lee Crosby and Mr. George Quabeck greatly aided the rabies work in this laboratory.

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1. Webster, L. T., *Am. J. Hyg.*, 1939, v30, 113.

2. TenBroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 523.