THE EFFECT OF SOME CHEMICALS ON PURIFIED INFLUENZA VIRUS*

By C. A. KNIGHT, Ph.D., and W. M. STANLEY, Ph.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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It is important in studies on the properties of influenza virus to know the effect of certain types of chemicals on the activity of the virus. In some cases it is desired to preserve maximum activity, while in others, such as the preparation of some vaccines, it is useful to know appropriate virucidal agents. Stock and Francis studied the inactivating effect of various soaps and organic acids on mouse lung suspensions of the PR8 and other strains of influenza virus (1). Other investigators have described the effects of miscellaneous agents such as phenol, formaldehyde, alum, calcium phosphate, and hydrogen peroxide on partially purified or mouse lung suspensions of virus (2–7). In the present investigation, tests were made of the effect of 20 different chemicals on the activity of purified preparations of the PR8 strain of influenza virus obtained by differential centrifugation.

Materials and Methods

Preparation of Virus.—0.2 ml. of a 10^{-4} to 10^{-7} dilution of allantoic fluid containing egg-adapted PR8 virus was inoculated into the peri-embryonic space of 9- to 10-day old chick embryos through a small hole above the air sac. After 48 hours at 37° the eggs were removed to a room held at 4° and chilled overnight. The allantoic fluids were then harvested, combined, and spun in a low-speed centrifuge. The clarified allantoic fluid was next centrifuged at 24,000 R.P.M. for 1½ to 2 hours. The pellets obtained were dissolved in 0.1 M phosphate buffer and returned to the high-speed centrifuge after removing insoluble matter in a low-speed centrifuge. The material obtained after two such cycles will be referred to as purified virus and was used in the chemical tests. Nitrogen analyses were made by the Nessler method from which virus protein was estimated by using the factor 10 (8). Ordinarily such preparations were found to possess a chicken red cell agglutination activity (CCA) of about 3800 units (9, 10) per mg. of protein and to have a 50 per cent infectivity end point in chick embryos (11) of 10^{-13} gm., but end points in the neighborhood of 10^{-14} gm. were occasionally observed.

Mixtures of Chemical and Virus.—Freshly prepared virus in 0.1 M phosphate buffer at pH 7 was well mixed with an equal volume of an aqueous solution of the chemical

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to be tested. The final concentration of protein was approximately 10^{-4} gm. per ml. in every case.

TABLE I

Effect of Chemicals on the PR8 Strain of Influenza Virus on Standing at 4° Chick Embryos—Red Cell Tests on Allantoic Fluids 48 Hours after Injection of Test Solutions

Chemical	0 days		10 days		17 days				27 days				35 days			
	$10^{-4.6}$	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
0.05 N Solutions of Chemical																
1. Iodine	0	0	0	0												
2. Cysteine‡	+	+	+	+	+	+	+	+	+	+	+	0				
3. Glucose	+	+	+	+	+	+	+	0	+	+	+	0				
4. Copper sulfate‡	+	0	0	0												
5. Silver nitrate‡	0	0	0	0												
6. Mercurochrome	+	+	0	0												
7. Formaldehyde	0	0	0	0												
8. Ammonium sulfate	+	0	+	+	+	+	+	0	+	0	0	0				
9. Sodium dodecyl sulfate‡	+	+	0	0												
10. Arginine	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+
Control (virus in 0.1 M phosphate)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
0.5 N Solutions of Chemical																
1. Iodine	0	0	0	0												
2. Cysteine§	+	+	0	0	0											
3. Glucose	+	+	+	+	+	+	+	0	+	+	+	0				
4. Copper sulfate‡	+	+	0	0												
5. Silver nitrate‡	0	0	0	0												
6. Mercurochrome	0	0	0	0												
7. Formaldehyde	0	0	0	0												
8. Ammonium sulfate	+	+	+	+	+	+	+	0	+	+	+	0				
9. Sodium dodecyl sulfate‡	+	+	+	0	0	0	0	0								
10. Arginine	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+

* Figures for test dilutions indicate approximate grams of protein per milliliter of test solution. Three eggs were used to test each dilution, and each egg was injected with ⅛ ml. Allantoic fluids from the three eggs were pooled for testing. + indicates presence of virus.

‡ Precipitates occurred in the virus-phosphate-chemical mixtures.

§ Solution became strongly acid.

Infectivity Tests.—Samples of mixtures of chemical and virus were removed immediately and at intervals thereafter and tested for virus activity in eggs and in mice. All solutions were kept at 4° between tests. Dilutions for the tests were made with sterile 0.1 M phosphate buffer at pH 7. For the tests in eggs, about 0.3 ml. of each dilution of the test fluid was injected into the allantoic sac of each of three 10-day old

embryos through a small hole above the air sac. The holes were then sealed with a colored collodion solution and the eggs incubated at 37° for 36 to 48 hours. At the end

TABLE II

Effect of Chemicals on the PR8 Strain of Influenza Virus on Standing at 4° Chick Embryos—Red Cell Tests on Allantoic Fluids 48 Hours after Injection of Test Solutions

Chemical	0 days		1 day		8 days			22 days				43 days		
	10^{-5}*	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-5}	10^{-6}	10^{-7}
0.05 N Solutions of Chemical														
11. Phemerol	0	0	0	0										
12. Roccal	0	0	0	0										
13. Calcium chloride‡	+	+	+	+		+	+		+		+		+	0
14. Sodium thiosulfate	+	+	+	+		+	+		+		+		+	+
15. Iodoacetamide	+	+	+	+		0	0							
16. Phenol	+	+	+	+		+	+	+		0		0	0	
17. Ascorbic acid	0	0	0	0	0	0								
18. Sulfathiazole sodium	+	+	+	+		+	0	+		+		+	0	
19. Urea	+	+	+	+		+	+		+		+		+	+
20. Thioglycolic acid§	+	0	0	0		0	0							
Control (virus in 0.1 M phosphate)	+	+	+	+		+	+		+		+		+	+
0.5 N Solutions of Chemical														
11. Phemerol	0	0	0	0										
12. Roccal	0	0	0	0										
13. Calcium chloride‡	0	0	+	0	0	0								
14. Sodium thiosulfate	+	+	+	+		+	+		+		+		+	+
15. Iodoacetamide	0	0	0	0										
16. Phenol	0	0	0	0										
17. Ascorbic acid§	0	0	0	0										
18. Sulfathiazole sodium	0	0	+	0	+	+		+		+		+	+	
19. Urea	+	+	+	+		+	+		+		+		+	+
20. Thioglycolic acid§	+	0	+	0	0	0								

* See Table I, footnote *.

‡ Precipitate of calcium phosphate was obtained.

§ The phosphate buffer failed to maintain the pH at 7 in the case of the 0.5 N ascorbic acid but did in the case of the 0.05 N. Both 0.5 and 0.05 N solutions of thioglycolic acid were acidic.

of this period, the eggs were chilled for several hours and the allantoic fluids were harvested and tested for virus hemagglutinins (9). Tests in mice were made by intranasal inoculation under light ether anesthesia of 0.05 to 0.1 ml. of virus solution. Mice which died were examined for typical pulmonary involvement and the surviving animals were sacrificed on the 10th day and autopsied. Those which died are listed in the tables as D with a subscript denoting the day of death. The degree of pulmo-

nary involvement in the animals surviving on the 10th day is indicated by numerals, with 4 = lung completely consolidated, 3 = ¾ consolidated, etc.

TABLE III

Effect of Chemicals on the PR8 Strain of Influenza Virus on Standing at 4°

Mouse Tests

Chemical	0 days	10 days	27 days	35 days		
	10^{-6}*	10^{-6}	10^{-6}	10^{-6}	10^{-7}	10^{-8}
0.05 N Solutions of Chemical						
1. Iodine	0, 0, 0	0, 0, 0				
2. Cysteine		D_8, D_8, D_9	D_7, D_8, D_9			
3. Glucose	D_7, D_8, D_8	D_6, 0, 0	D_8, D_9, 2			
4. Copper sulfate	0, 0, 0	0, 0, 0				
5. Silver nitrate	0, 0, 0	0, 0, 0				
6. Mercurochrome	0, 0, 0	0, 0, 0				
7. Formaldehyde	0, 0	0, 0, 0				
8. Ammonium sulfate	D_6, D_7, 0	±, 0, 0	0, 0, 0			
9. Sodium dodecyl sulfate	D_6, D_7, D_7	0, 0, 0				
10. Arginine	D_5, D_5, D_6	D_7, D_7, 3	D_5, D_6, D_7	D_5, D_5, D_6	D_7, D_9, 3	1, 1, 0
Control (virus in 0.1 M phosphate)	D_6, D_6, D_6	D_5, 2, 1	D_6, D_6, D_6	D_5, D_7, D_9	2, 1, 1	±, 0, 0
0.5 N Solutions of Chemical						
1. Iodine	0, 0, 0	0, 0, 0				
2. Cysteine	2, 2, 1	0, 0, 0				
3. Glucose	D_6, D_6, 2	D_6, D_6, 2	2, 1, 1			
4. Copper sulfate	0, 0, 0	0, 0, 0				
5. Silver nitrate	0, 0, 0	0, 0, 0				
6. Mercurochrome	0, 0, 0	0, 0, 0				
7. Formaldehyde	0, 0	0, 0, 0				
8. Ammonium sulfate	3, 2, 1	D_8, D_8, 2	D_8, 1, 0			
9. Sodium dodecyl sulfate	1, 1, ±	±, 0, 0				
10. Arginine	D_3, D_4, D_6	D_5, D_5, D_7	D_5, D_7, D_8	D_5, D_7, 1	D_9, 3, 0	±, 0, 0

* Figures for test dilutions indicate approximate grams of protein per milliliter of test solution. Three mice were used to test each dilution, and each mouse was inoculated with 0.05 to 0.1 ml.

EXPERIMENTAL

The strength of the reagents tested was put on a molecular size and active grouping basis by the use of solutions of fractional normality. This is believed to afford a more equitable comparison of the different reagents than could be obtained by the use of solutions containing a weight percentage of the reagents. In some cases the tests were complicated by the formation of precipitates, usually arising from reaction of the chemical and the phosphate buffer. In such instances, an attempt was made to obtain a uniform suspension of the precipitate in making dilutions for the tests in eggs and mice. It was difficult

TABLE IV

Effect of Chemicals on the PR8 Strain of Influenza Virus on Standing at 4°
Mouse Tests
0.05 N and 0.5 N Solutions of Chemical

Chemical	0 days		1 day		8 days				22 days			
	0.05 N	0.5 N	0.05 N	0.5 N	0.05 N		0.5 N		0.05 N		0.5 N	
	10^{-6}*	10^{-6}	10^{-6}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
11. Phemerol	0, 0, 0	0	0, 0, 0	0, 0, 0								
12. Roccal	1, 0, 0	0, 0	0, 0, 0	0, 0, 0								
13. Calcium chloride	D_6, 3, 1	1, 0, 0	D_7, D_7, D_8	1, 0, 0		D_9, 2, 1	0, 0, 0			0, 0, 0		
14. Sodium thiosulfate	D_6, D_7, D_8	D_6, D_7, D_8	D_6, D_6, D_8	D_6, D_7, D_9		D_8, D_9, 0		D_7, 2, 1		D_6, D_8, 3		2, 1, 0
15. Iodoacetamide	D_7, D_7, 2	±, ±, 0	2, 1, ±	0, 0, 0		0, 0, 0						
16. Phenol	D_7, D_9, 2	±, 0, 0	D_7, D_8, 3	2, 0, 0		1, 0, 0			0, 0, 0			
17. Ascorbic acid‡	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0							
18. Sulfathiazole sodium	D_6, D_7, D_7	0, 0, 0	1, 1, 1	1, 0, 0		1, 0, 0	0, 0, 0		0, 0, 0		0, 0, 0	
19. Urea	D_6, D_7, 3	D_6, D_6, D_7	D_6, D_7, D_7	D_5, D_7, 2		D_6, D_6, 2		D_6, 3, 1		D_9, 3, 1		D_7, 2, 2
20. Thioglycolic acid‡	1, 0, 0	1, 0, 0	0, 0, 0	1, 0, 0		0, 0, 0	0, 0, 0					
Control (virus in 0.1 M phosphate	D_4, D_8, D_9		D_6, D_6, D_7			D_6, D_7, D_8				D_6, D_9, 2		

* See footnote *, Table III.

‡ The phosphate buffer failed to maintain the pH at 7 in the case of the 0.5 N ascorbic acid but did in the case of the 0.05 N. Both 0.5 and 0.05 N solutions of thioglycolic acid were acidic.

to test the stronger solutions of phemerol and roccal in mice, since the chemicals frequently killed the mice when instilled into their lungs. The more dilute solutions, however, were administered without difficulty.

TABLE V

Effect of Chemicals on the PR8 Strain of Influenza Virus on Standing at 4°
Chick Embryos—Red Cell Tests on Allantoic Fluids 48 Hours after Injection of Test Solutions

Chemical	0 days		1 day		9 days				16 days			30 days		
	10^{-6}*	10^{-8}	10^{-6}	10^{-8}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-5}	10^{-6}	10^{-8}	10^{-5}	10^{-6}	10^{-8}
0.0005 N Solutions of Chemical														
Iodine	+	0	0	0	0		0							
Phemerol	+	+	+	0	+		0		0	0				
Mercurochrome	+	+	+	0	+		0		0	0				
Formaldehyde	+	+	+	+		+		+		+	0	0	0	
Ascorbic acid	+	+	+	+		+		+		+	+		+	+
0.005 N Solutions of Chemical														
Iodine	0	0	0	0	0		0							
Phemerol	+	0	+	0	+		0		0	0		0	0	
Mercurochrome	0	0	0	0	0		0							
Formaldehyde	+	+	0	0	0		0							
Ascorbic acid	+	+	+	+		+		+		+	+		+	0
Control (virus in 0.1 M phosphate)	+	+	+	+		+		+		+	+		+	+

Mouse Tests

0.0005 N and 0.005 N Solutions of Chemical

Chemical	0 days		1 day		9 days			
	0.0005 N	0.005 N	0.0005 N	0.005 N	0.0005 N		0.005 N	
	10^{-6}‡	10^{-6}	10^{-6}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
Iodine	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0		0, 0, 0	
Phemerol	1, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0		0, 0, 0	
Mercurochrome	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0		0, 0, 0	
Formaldehyde	D_6, D_6, D_9	3, 1, 1	D_6, D_6, D_7	0, 0, 0		0, 0, 0	0, 0, 0	
Ascorbic acid	D_5, D_6, D_7	D_6, D_7, 2	D_6, D_7, D_8	D_7, 2, 1		D_5, D_7, D_7		D_7, 1, 0
Control (virus in 0.1 M phosphate)	D_6, D_7, D_7		D_4, D_5, D_6			D_5, D_5, D_7		

* See Table I, footnote *.

‡ See Table III, footnote *.

The use of 0.1 M phosphate buffer maintained a pH and chemical environment known to be very favorable for the preservation of virus activity (12, 13). Hence, with the exception of the thioglycolic acid solutions and solutions of 0.5 N ascorbic acid and of cysteine which became strongly acid, the observed effects may be attributed to the chemicals tested. The results of the tests with 0.05 and 0.5 N chemicals are given in Tables I to IV. Some of the chemi-

cals used were found to inactivate the virus very rapidly. A few of these were retested at much lower concentrations, with the results shown in Table V.

Since phenolized viruses are sometimes used as vaccines, it was considered of interest to make additional tests on phenol-virus mixtures. For this purpose, purified virus in 0.1 M phosphate buffer was mixed with an equal volume of phenol solution to give final mixtures containing about 10^{-4} gm. of protein per ml. and concentrations of phenol of 0.1 per cent and 0.5 per cent (0.01 and 0.05 N, respectively). Immediately, and at weekly intervals thereafter, samples were removed and diluted for infectivity tests in 10-day chick embryos. Between tests, the solutions were kept at 4°. The results of the tests are presented in Table VI.

TABLE VI

Effect of 0.1 Per Cent and 0.5 Per Cent Phenol on the PR8 Strain of Influenza Virus on Standing at 4°

Chick Embryos—Red Cell Tests on Allantoic Fluids 48 Hours after Injection of Test Solutions

	Days							
	0	7	14	21	28	35	42	79
0.1 Per Cent Phenol								
10^{-6}*	+	+	+	+	+	+	+	+
10^{-8}	+	+	+	+	+	+	+	+
10^{-10}	+	+	+	+	+	+	+	+
10^{-12}	+	+	+	+	+	0	0	0
0.5 Per Cent Phenol								
10^{-6}	+	+	+	+	+	+	+	+
10^{-8}	+	+	+	+	+	+	+	0
10^{-10}	+	+	+	0	0	0	0	0
10^{-12}	+	+	0	0	0			

* See Table I, footnote *.

DISCUSSION

The chemicals which have a direct inactivating effect on viruses can generally be classified as protein-precipitating agents, oxidizing agents, and strong acids or alkalis (14). Reducing agents, and cysteine in particular, are known to stabilize preparations of tomato-spotted wilt, herpes, and equine encephalomyelitis viruses (15–18).

In the present investigation, salts of heavy metals were found to inactivate the virus rapidly, silver nitrate acting a little more quickly than copper sulfate. Also, mercurochrome, even in minute concentrations, was observed to inactivate the virus readily. It may be noted that mercurochrome was one of the

few chemicals which in very small amounts was found by Schultz and Robinson to inactivate poliomyelitis virus (19).

Iodine, as a typical strong oxidizing agent, destroyed virus infectivity promptly, even in 0.0005 N solution. Iodoacetamide, which may or may not act as a specific reactant for protein SH groups (20), was found to inactivate the virus at once in 0.5 N solution and more slowly in 0.05 N solution. However, since a starch test on the iodoacetamide-virus mixture revealed the presence of free iodine, in the present tests it is impossible to separate the iodine effect from any specific action which the iodoacetamide may have possessed.

Virus in solution with the reducing agents, glucose and sodium thiosulfate, appeared to be about as stable as virus in 0.1 M phosphate buffer, although in the case of glucose there was some indication of inactivation at 17 days. Tests with the reducing agents, cysteine hydrochloride, thioglycolic acid, and ascorbic acid, were largely invalidated since the phosphate buffer did not maintain the pH at a point where the virus was stable. Two exceptions were the 0.05 N solutions of cysteine and of ascorbic acid. The pH of the cysteine solution approached neutrality with the result that the cysteine was oxidized to cystine. This was shown by pH measurements and by the fact that the solution containing 0.5 N cysteine gave a strongly positive nitroprusside test at the end of the test period, while the 0.05 N solution gave no color until sodium cyanide was added. Hence, the test starting with 0.05 N cysteine was actually a test of cystine, and the virus proved to be quite stable in the presence of this chemical. The pH of the 0.05 N solution of ascorbic acid was between 6 and 7, and yet it caused a tremendous inactivation of the virus from the outset. The reason for this result is not yet clear.

The detergents, phemerol[1] and roccal, proved to be highly virucidal, causing immediate inactivation at 0.5 and 0.05 N concentrations and complete inactivation in 0.005 and 0.0005 N solutions after 2 weeks. From these results it is apparent that, if such detergents are used as bactericidal agents in the presence of virus, they must be used only in very small concentrations such as reported by Krueger and associates (21). At the concentrations tested, sodium dodecyl sulfate completely inactivated the virus only upon standing for a week or more. The virus appeared to be as stable in 0.5 N urea as in 0.1 M phosphate buffer.

0.5 and 0.05 N solutions of formaldehyde caused immediate and complete inactivation of the purified virus, as many others have noted in tests with crude suspensions of virus. Even the 0.0005 N solution proved to be virucidal after 30 days.

0.05 N solutions of ammonium sulfate and of calcium chloride had a slight inactivating effect, as did 0.5 N ammonium sulfate. There appeared to be considerable inactivation in the case of 0.5 N calcium chloride, but it seems

[1] The authors are indebted to Parke, Davis and Company for a generous supply of phemerol.

possible that this may have been due, at least in part, to the heavy precipitate of calcium phosphate which is known to adsorb and occlude the virus (7). The precipitate was much less abundant in the case of the 0.05 N solution which proved quite active.

The amino acid, arginine, appeared to help stabilize solutions of the virus in 0.1 M phosphate buffer. Both the chick embryo and mouse tests indicated that, at the end of 35 days, the solution containing virus, phosphate buffer, and 0.05 N arginine was more active than the control virus in phosphate buffer alone. However, this small difference has not yet been confirmed in tests with larger numbers of embryos and mice.

Sulfathiazole sodium was tested as a typical sulfa drug. Some inactivation of the virus was observed, but it was by no means complete, for even in the presence of a 0.5 N solution of drug (about 15 per cent) there was considerable virus activity left at 43 days. These results may help to explain the observation of Coggeshall and Maier that none of a group of sulfa drugs was effective in delaying or preventing the development of influenza in mice when the latter were injected with PR8 virus (22).

The 0.5 N solution of phenol inactivated the virus promptly, but the 0.05 N solution required 3 weeks or more to accomplish the same effect. The more detailed study made with final concentrations of phenol of 0.1 and 0.5 per cent (0.01 and 0.05 N) indicated that there was but very little loss of activity in 0.5 per cent phenol until after 1 week and in 0.1 per cent phenol only after 1 month.

SUMMARY

A study was made of the effect of 0.05 and 0.5 N solutions of 20 different chemicals on the activity of purified PR8 influenza virus in 0.1 M phosphate buffer. It was found by tests in chick embryos and in mice that virus activity was destroyed by strong oxidizing agents such as iodine, by salts of heavy metals, by mercurochrome, by formaldehyde, and by the detergents phemerol, roccal, and sodium dodecyl sulfate. Reducing agents appeared to have little if any inactivating effect with the exception of 0.05 N ascorbic acid. At the concentrations tested, sulfathiazole sodium exerted only a weak inactivating effect. 0.5 N phenol inactivated the virus promptly, but solutions of the strength more commonly used for bactericidal purposes were only weakly virucidal. The virus appeared relatively unaffected by glucose, ammonium sulfate, calcium chloride, sodium thiosulfate, and arginine.

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