INFLUENCE OF pH AND OF CERTAIN OTHER CONDITIONS ON THE STABILITY OF THE INFECTIVITY AND RED CELL AGGLUTINATING ACTIVITY OF INFLUENZA VIRUS*

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A factor of considerable importance to the stability of the infectivity of influenza virus is pH. Andrewes and Smith found in preliminary experiments that the infectivity of the W.S. strain of influenza virus was fairly stable between pH 7 and 9 but that it was unsafe to keep the virus very long on the acid side of pH 7 (1). Ostrovskaya and coworkers reported that the influenza virus which they studied was most stable at pH 7.0–7.5 (2). Stock and Francis found the PR8 strain to be most stable at pH 7.0 (3).

The effect of pH upon the chicken red blood cell agglutinating (CCA) activity of preparations of influenza virus has not, however, been tested. Since the measurement of CCA activity as well as of infectivity can be used as a biological test for the characterization of influenza virus (4), it is of importance to establish conditions of maximum stability for both. Such a study would possess the added interest that the determination of the relative pH stabilities of the different biological activities might yield information bearing upon the question of whether or not both activities arise from one and the same particle. Accordingly, a detailed investigation has been made of the pH stability of strains of influenza virus with respect both to infectivity and to CCA activity.

The present studies were carried out with preparations of PR8, Lee, and swine viruses concentrated and purified from the allantoic fluid of infected chick embryos by means of differential centrifugation. Previous investigators (1–3) employed suspensions or filtrates of infected mouse lungs. In order that the importance of temperature to the stability of the virus might be determined, certain of the experiments were carried out at room temperature and others at 4° C. Observations also were made on the effect of the composition of the buffers and of the concentration of the virus protein on the stability of the virus.

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Materials and Methods

Source of Virus.—PR8 and Lee strains of influenza virus were supplied by Dr. T. Francis, Jr. Swine influenza virus, strain 15 of Dr. R. E. Shope, was supplied by Dr. F. B. Bang. Centrifugally purified preparations of the various strains were obtained by methods described in earlier publications from this laboratory (5, 6). It is not presumed that completely pure virus was obtained in any case, but simply that considerable concentration and a large measure of purification were effected.

In order to remove electrolyte and most of the buffering material from preparations of purified virus, the pellets of virus obtained from high-speed centrifugation were dissolved in distilled water and were then immediately recentrifuged at high speed. Because of the instability of the infectivity and the CCA activity of the virus in a medium of distilled water (7, 8), the above procedure probably led to a partial loss in biological activity. The final pellets were dissolved in distilled water, and after estimates of protein concentration were made, suitable aliquots were added to the buffer solutions.

Preparation of Mixtures of Virus and Buffers.—Tenth molar citrate buffers at pH 3.0 and 4.0, M/15 phosphate buffers at pH 5.0–9.0, and M/10 glycine-NaCl buffers at pH 7.0–11.0 were made up according to the directions of Clark (9). Tenth molar phosphate buffer at pH 7.0 and buffer mixtures containing approximately M/30 phosphate and M/20 glycine-NaCl at pH 7–10 also were prepared. To 9 parts by volume of freshly prepared buffer was added 1 part of an aqueous solution containing approximately 1 mg. of ultracentrifugally purified virus per cc. Strict precautions for sterility were not found essential.

Virus Infectivity.—For the measurements of virus infectivity, aliquots of the test solutions were diluted serially at tenfold intervals, usually with sterile 0.1 M phosphate buffer at pH 7, and in a few instances with sterile 0.85 per cent saline. Young mice, 3 to 4 weeks of age, were anesthetized with ether and were given intranasally about 0.05 cc. of suitable dilutions of virus. Three to five mice were employed for each dilution to be tested. Mice which died were examined for typical pulmonary involvement and the surviving animals were sacrificed on the 10th day and were autopsied. Those which died of influenza are listed in the tables as D with a subscript denoting the day of death. The degrees of pulmonary involvement are indicated by numerals, with 4 representing lungs completely consolidated; 3, ¾ consolidated, etc. Mice which were missing at autopsy or which died from causes other than influenza are listed as M.

CCA Activity.—Measurements of CCA activity were carried out by a modification of the method of Hirst and Pickels (10, 11). A reproducible virus standard for CCA activity (11) was not available until after most of the present study was completed, hence, the CCA titers reported in the present paper are not standardized except where indicated. Differences or lack of differences in CCA titers could be judged quite certainly, however, by comparisons of determinations carried out simultaneously on different unknowns and by observing trends over sufficiently long periods of time.

When the solutions to be tested were not at pH 7, they were first neutralized, or, as was found more practical, the serial dilutions for the tests were carried out in 0.1 M pH 7 phosphate buffer which effectively minimized differences in pH.

Estimation of Protein.—Protein determinations were carried out by the Nessler method described elsewhere (5).

EXPERIMENTAL

Stability of PR8 Virus

Infectivity and CCA Activity of PR8 Virus Kept at 23° C. in Various Buffers at pH 3–11.—In experiments carried out at room temperature, 23° C., it was

observed that at pH 3, 4, and 5 the infectivity of PR8 influenza virus was lost within 1 hour. It was noted further that at pH 4 and 5 a considerable amount of precipitate separated from solution, suggesting the isoelectric precipitation of the virus. Ultracentrifugally isolated virus thus appeared to be somewhat less stable at the above hydrogen ion concentrations than did the virus when present in mouse lung suspension (3). The results obtained at pH 6–9 in phosphate buffer and at 10–11 in glycine-NaCl buffer are shown in Table I. It can be concluded that the influenza virus was much more stable at pH 6 or 7 than at any higher pH, since, within the range of pH 8–11, prac-

TABLE I

Infectivity of PR8 Influenza Virus Kept at 23° C. at pH 6–11

Time	Dilution of virus preparation	Infectivity for mice					
		pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
	gm. per cc.						
1 hr.	10^{-6}	D_4, D_4, D_6	D_7, D_8, M	D_6, D_6, D_7	D_6, D_8, D_8	D_6, D_6, D_7	D_5, D_6, M
	10^{-8}	1, 1, 0	1, 0, 0	1, 1, 1	1, 0, 0	0, 0, 0	1, 1, 0
1 day	10^{-6}	D_7, D_7, D_7	D_7, D_8, D_8	D_8, 1, 1	2, 1, 1	D_{10}, 3, 1	D_9, D_9, D_9
	10^{-8}	1, 1, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3 days	10^{-6}	D_9, D_9, 2	D_9, 1, 1	1, 0, 0	0, 0, 0	1, 0, 0	1, 0, 0
	10^{-8}	1, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
10 days	10^{-5}	D_4, D_4, D_4	D_4, D_5, D_5	1, 1, 0	D_{10}, 0, 0	0, 0, 0	D_{10}, 0, 0
	10^{-7}	1, 1, 0	D_{10}, 1, 0	0, 0, 0	1, 0, 0	D_{10}, 0, 0	D_{10}, 0, 0
30 days	10^{-4}	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	10^{-6}	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

tically all virus infectivity disappeared after 3 days, whereas, at pH 6 or 7, around 10 per cent of the infectivity still remained after 10 days. It appears worthy of special note that the virus also was much more stable in relatively strongly alkaline solution, for example, at pH 10 or 11, than it was in weakly acid solution such as that at pH 5. Even under optimum conditions of pH, however, over 99 per cent inactivation occurred after 30 days.

After standing at room temperature for 30 days, the various test solutions were adjusted to pH 7 and rough measurements of CCA activity were carried out. The starting preparation of virus contained 5,000 CCA units per mg. of nitrogen. The test solutions, originally at pH 3–11, exhibited CCA activities of <30, <30, <30, 400, 4,000, 3,000, 4,000, 7,000, and 2,000 CCA units per mg. of nitrogen, respectively. It is apparent from these results that the losses

in virus infectivity observed at pH 5 or below were accompanied by marked losses in agglutinating power, whereas the losses in infectivity at pH 8 or above were accompanied by relatively little change in agglutination titer.

Infectivity and CCA Activity of PR8 Virus Kept at 4° C. in Phosphate Buffer at pH 5.5–7.0.—It was of particular interest that at pH 6 and 7, where the virus infectivity persisted the longest, significantly different CCA activities

TABLE II

Infectivity and CCA Activity of PR8 Influenza Virus Kept at 4°C. in Phosphate Buffer at pH 5.5–7.0

Time	Dilution of virus preparation	Infectivity for mice			
		pH 5.5	pH 6.0	pH 6.5	pH 7.0
	gm. per cc.				
1 day	10^{-6}	2, 2, 1	D_8, 3, M	D_8, D_8, D_9	D_7, D_9, 3
	10^{-8}	0, 0, 0	2, 2, 1	±, ±, 0	1, 1, 1
8 days	10^{-6}	0, 0, 0*	D_7, ±, 0	D_6, D_8, D_9	D_6, D_7, D_7
	10^{-8}		±, 0, 0	0, 0, 0	0, 0, 0
24 days	10^{-4}	±, 0, 0	1, 1, 1	D_5 D_6 D_8	D_4, 1, 1
	10^{-6}		0, 0, 0	D_7, D_8, D_8	D_7, 0, M
32 days	10^{-4}	0, 0, 0	±, 0, 0	D_5, D_5, D_6	D_2, D_2, D_5
	10^{-6}		D_{10}, 0, 0	1, ±, ±	1, ±, ±
		CCA activity			
1 day		90‡	2,900	2,800	4,300
5 days			500	3,500	5,000
23 days			80	1,300	2,900
37 days			130	1,100	2,600

* Results obtained with 10^{-4} gm. per cc. in inoculum.

‡ Units of CCA activity per mg. of nitrogen.

were obtained, for in the former case a markedly lowered, yet definitely positive, titer was exhibited. It seemed worthwhile, therefore, to investigate in greater detail within the pH range of 5.5–7.0 in phosphate buffer the correlation between the two types of biological activities of the influenza virus. In order to test at the same time the importance of temperature to the stability of the virus, the present experiments were carried out at 4° C. The results of the infectivity measurements, shown in the top part of Table II, indicate a maximum stability of the virus at pH 6.5 and 7.0 with a marked diminution in stability toward the acid side of the pH range. The data, when compared with those of Table I, also demonstrate that the infectivity of the virus is maintained much better

at the lower temperature, namely 4° C. The higher stability of the virus at 4° has been confirmed in other experiments.

From the results of the agglutination tests presented in the bottom part of Table II, it can be concluded that the CCA activity was most stable at pH 7.0 and increasingly unstable at more acid hydrogen ion concentrations. The loss in CCA activity at pH 5.5 and 6.0 accompanied the loss in infectivity of the virus under the same conditions.

TABLE III

Infectivity and CCA Activity of PR8 Influenza Virus Kept at 4°C. in Composite Phosphate-Glycine-NaCl Buffer at pH 7.0–9.1

Time	Dilution of virus preparation	Infectivity for mice			
		pH 7.0	pH 7.9	pH 8.6	pH 9.1
	gm. per cc.				
1 mo.	10^{-5}	D_3, D_4, 2	D_2, D_3, D_4	D_3, D_4, D_6	D_5, D_5, D_6
	10^{-6}	D_4, D_4, D_5	D_5, D_9, 2	D_7, D_9, 2	2, 2, 2
	10^{-7}	2, 1, 0	2, 1, ±	1, ±, 0	1, 1, 0
	10^{-8}	1, 1, ±	1, 1, ±	0, 0, 0	1, 0, 0
	10^{-9}	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3 mos.	10^{-4}	D_3, D_3, D_5	D_3, D_5, D_5	2, 1, ±	0, 0, 0
	10^{-5}	D_3, D_3, D_5	D_5, D_7, D_9	1, ±, 0	0, 0, 0
	10^{-6}	D_5, D_5, D_7	3, 3, 1	0, 0, 0	0, 0, 0
	10^{-7}	D_9, D_9, 3	±, ±, 0		
	10^{-8}	D_5, 2, 1	0, 0, 0		
		CCA activity			
4 days		14,000*	13,000	11,000	3,300
1 mo.		9,700	11,000	6,600	3,200
3 mos.		11,000	12,000	6,700	3,100

* Units of CCA activity per mg. of nitrogen.

Infectivity and CCA Activity of PR8 Virus Kept at 4° C. in Composite Phosphate-Glycine-NaCl Buffer at pH 7.0–9.1.—It remained of importance to establish more precisely the effect of pH upon the CCA activity of PR8 influenza virus in solutions more alkaline than pH 7. In order to lessen possible complications which might arise from the use of buffers of different composition, *i.e.* phosphate buffer and glycine-NaCl buffer, composite buffers made up with both constituents were employed. The mixtures of virus and buffer were allowed to stand at 4° C. and tests for CCA activity were carried out at intervals of 4 days, 1 month, and 3 months. Tests for infectivity also were carried out at the 1 and 3 month intervals. The final results are presented in Table III. Within the limits of error of the various measurements, the

following conclusions can be made from the data. The stability of the CCA activity of PR8 virus in the composite buffer is essentially the same at pH 7.0 and pH 7.9 but is progressively lower at more alkaline reactions. The infectivity of the virus is appreciably more stable at pH 7.0 than at pH 7.9. At pH 8.6 or 9.1, the CCA activity of the virus is rapidly reduced to somewhat lower values which then appear to remain more or less constant, whereas under the same conditions the infectivity continues to decrease.

Stability of Lee Virus

Infectivity and CCA Activity of Lee Virus Kept at 4° C. in Phosphate Buffer at pH 5.4–7.9.—The infectivity of the Lee strain of influenza virus kept at 4° C. in phosphate buffers over the range pH 5.4–7.9, is shown by the data presented in Table IV from which it can be concluded that the most favorable hydrogen ion concentration was provided at the highest pH employed. Agglutination tests carried out at the end of 3 months revealed an almost complete loss of CCA activity in all samples.

TABLE IV

Infectivity of Lee Influenza Virus at 4°C. in Phosphate Buffer at pH 5.4–7.9

Time	Dilution of virus preparation	Infectivity for mice			
		pH 5.4	pH 6.4	pH 7.1	pH 7.9
	gm. per cc.				
1 hr.	10^{-7}	D_9, D_9, +	D_6, D_6, ±	D_6, D_8, D_9	D_5, D_5, D_6
	10^{-8}	++, +, +	D_7, D_8, D_8	D_7, D_8, D_{10}	D_8, D_8, +
1 day	10^{-7}	0, 0, 0	D_7, D_7, D_7	D_6, D_7, 2	D_8, D_8, 0
	10^{-8}	±, 0, 0	D_9, D_{10}, ±	D_9, D_{10}, 2	2, 1, ±
8 days	10^{-7}		D_8, D_8, D_8	3, 1, 0	D_7, ±, 0
	10^{-8}		2, 2, 1	2, ±, 0	3, 2, 0
12 days	10^{-6}		1, 0, 0	2, 1, ±	D_9, 2, 1
	10^{-7}		0, 0, M	1, 0, 0	±, ±, 0
32 days	10^{-4}		0, 0, 0	1, ±, 0	D_5, D_9, 2
	10^{-5}		0, 0, 0	0, 0, 0	2, 1, ±

Infectivity and CCA Activity of Lee Virus Kept at 4° C. in Composite Phosphate-Glycine-NaCl Buffer at pH 7.0–9.2.—It appeared necessary to test the persistence of the infectivity of the Lee virus under still more alkaline conditions and to follow more closely the changes in CCA activity. Composite buffers containing both phosphate and glycine-NaCl buffers were employed in these experiments. Mixtures of buffer and virus were prepared and were allowed

to stand at 4° C. The data obtained from infectivity and CCA measurements are presented in Table V. In contrast to the results obtained in phosphate buffer it appears that in a mixture of phosphate and glycine buffers the infectivity of the Lee virus was as stable at pH 7 as at pH 7.9. However, the agglutination tests indicate that pH 7.9 provided the optimum hydrogen ion concen-

TABLE V

Infectivity and CCA Activity of Lee Influenza Kept at 4° C. in Composite Phosphate-Glycine-NaCl Buffers at pH 7.0–9.2

Time	Dilution of virus preparation	Infectivity for mice			
		pH 7.0	pH 7.9	pH 8.6	pH 9.2
	gm. per cc.				
1 day	10^{-7}	D_6, D_6,D_7, D_8,D_9	D_8,D_9,D_{10},3, 1	D_6, D_7,D_8 D_9,0	D_8, D_{10},3, 3, ±
	10^{-8}	D_7, D_9,D_9, 2, 0	D_6,D_8,D_8, 3, 3	D_9, D_9,1, 1, M	3, 2, 2, 1, 1
16 days	10^{-6}	D_4, D_4,D_5, D_5,D_{10}	D_2,D_5,±, 0, 0	D_9, D_9,D_9,3, 3	0, 0, 0, 0, 0
	10^{-7}	D_9, 3, 3, 2, 2	1, 1, ±, 0, 0	2, 1, 1, 1, 1	±, 0, 0, 0, 0
1 mo.	10^{-5}	D_4, D_4,D_9	D_4,D_5,2	D_6, D_9,3	±, 0, 0
	10^{-6}	D_5, D_6,3	D_6,D_7,D_7	±, 0, 0	0, 0, 0
	10^{-7}	3, 1, ±	2, 1, ±	±, 0, 0	0, 0, 0
	10^{-8}	3, 2, 0	0, 0, 0	0, 0, 0	0, 0, 0
3 mos.	10^{-4}	D_5, D_5,D_6	D_5,D_5,D_8	0, 0, 0	0, 0, 0
	10^{-5}	D_7, D_9,±	D_7,D_8,±	0, 0, 0	0, 0, 0
	10^{-6}	2, 1, 1	1, 1, 0	0, 0, 0	0, 0, 0
	10^{-7}	±, 0, 0	0, 0, 0		
		CCA activity			
4 days		2,700*	3,100	1,500	1,300
1 mo.		5,800	4,300	2,900	1,100
3 mos.		720	4,000	2,700	1,900
		(2,600)‡	(3,200)‡		

* Units of CCA activity per mg. of nitrogen.

‡ Results obtained in duplicate test.

tration for the preservation of CCA activity. In a duplicate experiment, also presented in Table V, the results of the agglutination tests revealed less marked differences at pH 7 and pH 7.9. It can be concluded that the data as a whole, on the Lee virus, indicate a tendency for this strain to be unstable at the lower pH.

Infectivity and CCA Activity of Lee Virus in Different Buffer Media at the Same pH.—It was noted in the work just described that both the infectivity

and the CCA activity of Lee virus were stable for longer periods of time in the composite buffer than in phosphate buffer alone. In order to establish

TABLE VI

Infectivity and CCA Activity of Lee Influenza Virus Kept at 4°C. in Different Buffer Media

Buffer	Time	Infectivity for mice			CCA activity	
		Dilution of virus preparation	pH 7*	pH 9	pH 7	pH 9
		gm. per cc.				
Phosphate-Glycine-NaCl	1 mo.	10^{-6}	D_5, D_6, D_8	D_9, 3, 0	13,000‡	6,300
		10^{-7}	D_8, D_9, 2	1, ±, ±		
		10^{-8}	2, 1, ±	1, 1, 0		
	2 mos.	10^{-6}	D_8, D_8, 0	1, 0, 0	12,000	5,100
		10^{-7}	D_8, 3, 2	±, ±, 0		
		10^{-8}	1, 1, ±	0, 0, 0		
	3 mos.	10^{-4}	D_8, 1, ±	±, ±, ±	11,000	3,500
		10^{-5}	D_9, 2, 2	0, 0, 0		
		10^{-6}	0, 0, 0	D_{10}, ±, ±		
Glycine-NaCl	1 mo.	10^{-4}	D_8, D_6, D_6	D_5, D_6, D_7	12,000	11,000
		10^{-5}	D_6, D_8, D_8	±, ±, ±		
		10^{-6}	D_7, 3, 3	±, 0, 0		
		10^{-7}	±, 0, 0	0, 0, 0		
	2 mos.	10^{-4}	D_8, D_5, D_5	1, ±, 0	10,000	8,800
		10^{-5}	1, ±, 0	0, 0, 0		
		10^{-6}	2, 1, 1	0, 0, 0		
	3 mos.	10^{-4}	0, 0, 0	0, 0, 0	7,800	8,600
Phosphate	1 mo.	10^{-4}	±, ±, 0	1, ±, ±	5,100	13,000
		10^{-5}	0, 0, 0	±, ±, 0		
	2 mos.				<1,300	7,400
	3 mos.				<1,300	6,100

* These pH values represent hydrogen ion concentrations at the beginning of the experiments. At the end of the experiments it was found that all test solutions except the pH 7 phosphate solution tended to approach intermediate pH values of 7.5–8.

‡ Units of CCA activity per mg. of nitrogen.

the relative importance of different buffers in maintaining the infectivity and CCA activity of Lee influenza virus, further tests were made in which the composite buffer, the glycine-NaCl buffer, and the phosphate buffer were

employed as separate buffer media. The various mixtures of buffer and virus were prepared at pH 7 and pH 9 and were allowed to stand at 4° C. The results obtained in measurements of mouse infectivity and CCA activity carried out during the first 2 weeks showed rather small differences in the activities of the various test solutions with the exception of the pH 7 phosphate solution in which the virus lost considerable infectivity. The differences obtained at intervals of 1, 2, and 3 months, however, were more striking, as is shown by the data presented in Table VI. The results indicate that the infectivity of the virus possessed the greatest stability in the composite phosphate-glycine-NaCl buffer, an intermediate stability in the glycine-NaCl buffer, and the lowest stability in the phosphate buffer. This finding held true both at pH 7 and pH 9. The same order in relative stabilities also was obtained for the CCA activity of the virus at pH 7, although at pH 9 the results were irregular. It is important to note, however, that the CCA activity of the Lee virus in phosphate buffer was much more stable at pH 9 than at pH 7. The low stability of the CCA activity of Lee influenza virus at pH 7 is in marked contrast with the high degree of stability of the PR8 strain under the same conditions.

Stability of Swine Virus

Infectivity and CCA Activity of Swine Virus Kept at 4° C. in Phosphate Buffer at pH 5.4–8.—Preliminary studies on the stability of swine virus in phosphate buffer revealed a very great loss of infectivity within 1 hour at pH 5.4 with a more gradual loss of infectivity at pH 6.1. The results obtained at pH 7 and 8 were somewhat irregular but indicated approximately equivalent and maximum stabilities under these conditions. Agglutination tests carried out after 3 months showed over 90 per cent loss in CCA activity at pH 5.4 and 6.1 but complete stability at pH 7 and 8.

Infectivity and CCA Activity of Swine Virus Kept at 4° C. in Composite Phosphate-Glycine-NaCl Buffer at pH 7.0–9.2.—A more complete study was next carried out in the composite phosphate-glycine-NaCl buffers described earlier. The results, which are presented in Table VII, demonstrate that the infectivity and CCA factors both possessed maximum stabilities within the range of pH 7.0–7.9, with progressively diminishing stabilities at higher alkalinities.

Effect of Concentration of Virus on Stability

Because of the fact that the high specific biological activities of the viruses permitted a saving of material, all of the experiments on the stability of virus strains which have been described were carried out with low concentrations of virus protein, namely, 0.1 mg. per cc. A number of observations made from time to time, however, suggested that the viruses might be appreciably less stable in dilute solution than when more concentrated. In an earlier report it has been demonstrated that preparations of PR8 influenza virus

maintain constant CCA activity over a period of several months (11) at 4° C. These particular stability tests were carried out with concentrated solutions of virus. Recently, in putting the earlier findings into practice, a sample of a virus preparation containing 5 mg. of virus protein per cc. was diluted to 0.1 mg. per cc., and the stock solution thus prepared was stored at 4° C. and employed as a standard in the CCA test. Such a stock solution, however, was found gradually to lose CCA activity. At weekly intervals the CCA

TABLE VII

Infectivity and CCA Activity of Swine Influenza Virus Kept at 4°C. in Composite Phosphate-Glycine-NaCl Buffer at pH 7.0–9.2

Time	Dilution of virus preparation	Infectivity for mice			
		pH 7.0	pH 7.9	pH 8.6	pH 9.2
	gm. per cc.				
1 day	10^{-7}	D_6, D_7, D_7, 2, 1	D_6, D_7, D_8, D_8, D_8	D_6, D_9, D_{10}, D_{10}, 3	D_7, D_8, 1, 1, M
	10^{-8}	D_8, 1, 1, ±, 0	2, 1, 1 1, 1	1, 1, 1, 1, 1	1, ±, ±, 0, 0
16 days	10^{-7}	D_6, D_6, D_7, 1, 1	D_9, D_{10}, 3, 1, ±	D_9, 1, ±, ±, 0	2, 1, 1, 1, 1
	10^{-8}	1, 1, 0, 0, 0	2, 2, 1, ±, ±	D_9, 1, 1, ±, ±	1, 1, 1, 1, 0
1 mo.	10^{-5}	D_4, D_4, D_5,	D_5, D_6, 1	D_4, D_5, +	D_7, D_9, 1
	10^{-6}	D_5, D_5, D_6	D_5, D_6, D_6,	2, 2, 1	1, 1, ±
	10^{-7}	2, 1, ±	2, 1, ±	±, 0, 0	0, 0, 0
	10^{-8}	3, 2, ±	D_{10}, ±, 0	0, 0, 0	0, 0, 0
3 mos.	10^{-4}	D_5, D_5, M	D_5, D_5, 3	1, ±, ±	1, ±, ±
	10^{-5}	D_7, D_9, ±	D_6, D_6, 3	±, 0, 0	±, 0, 0
	10^{-6}	D_6, D_8, ±	D_9, 3, ±	D_9, ±, 0	0, 0, 0
	10^{-7}	2, 1, ±	1, ±, ±	D_8, 0, 0	
	10^{-8}	2, 1, ±	1, 0, 0	0, 0, 0	
		CCA activity			
4 days		7,800	7,900	6,200	8,000
1 mo.		5,800	4,700	2,600	1,500
3 mos.		7,700	10,000	4,600	1,300

activities of aliquots of the dilute stock solution were compared with that of the original concentrated solution. The percentage losses observed in nine such experiments were 5, −8, 14, 13, 11, 20, 28, 10, and 6, respectively, corresponding to an average loss of 12 per cent per week. While the diluted stock sample showed losses in CCA activity over 1 week's time, the concentrated starting solution showed no significant change in activity. The importance of storing PR8 virus in solutions more concentrated than 0.1 mg. per cc. was thus clearly established.

The problem of the storage of Lee virus, however, was not yet satisfactorily solved. The use of composite buffers containing glycine, although serving

well to preserve the virus activity, possessed the obvious disadvantage that measurements of protein nitrogen were complicated by the presence of glycine nitrogen. The finding that PR8 virus was more stable in concentrated than in dilute solution suggested that the same condition might be obtained with the Lee strain. It also seemed possible that, since the PR8 virus was much more stable even in dilute solution in 0.1 M phosphate buffer than the Lee virus under the same conditions, the presence of the former might augment the stability of the latter.

TABLE VIII

Infectivity and CCA Activity of Lee Influenza Virus, PR8 Influenza Virus, a Mixture of Lee and PR8 Viruses, and a Mixture of Lee Virus with Ultraviolet-Inactivated PR8 Virus, at 2 Mg. Per Cc. When Kept in Phosphate Buffer at pH 7

Time	Dilution of virus preparation	Infectivity for mice			
		Lee	PR8	Lee and PR8	Lee and U. V. PR8
	gm. per cc.				
1 day	10^{-7}	D_6, D_6, D_7, D_8, D_8	D_5, D_8, D_6, 1, M	D_6, D_6, D_8, D_8, 0	D_6, D_7, D_8, D_{10}, 1
	10^{-8}	D_9, D_9, D_{10} 1, ±	D_6, D_8, D_8, 3, 3	D_7, D_7, D_7, D_9, D_9	D_9, D_9, 3, 3, 3
	10^{-9}	2, 2, 1, 1, ±	1, 1, ±, ±, ±,	3, 3, ±, ±, 0	2, 1, ±, ±, 0
8 days	10^{-7}	D_5, D_8, D_6, D_7, D_8	D_4, D_6, D_6, D_6, D_7	D_5, D_5, D_6, D_6, D_7	D_8, D_6, D_7, D_7, D_7
	10^{-8}	D_7, D_8, D_8, D_8, 1	D_6, D_8, 3, 3, 2	D_6, D_6, D_9, D_9, D_9	D_8, D_8, D_9, 2, 2
	10^{-9}	D_7, D_9, 2, 1, ±	3, 1, 1, ±, ±	D_8, 3, 2, 2, 1	2, ±, ±, ±, 0
2 mos.	10^{-7}	D_7, D_7, D_7, D_7, D_7	D_6, D_6, D_6, D_7, 2	D_6, D_7, D_7, D_7, D_8	D_6, D_7, D_7, D_7, D_{10}
	10^{-8}	D_7, D_8, D_{10}, 2, 1	D_7, D_7, D_8, D_9, 3	D_7, D_9, 3, 2, 1	D_7, D_9, 2, 2, 1
	10^{-9}	2, 2, 1, ±, 0	D_9, 2, 2, 2, 1	D_9, 3, 2, 2, 1	2, 2, 2, 1, ±
		CCA activity			
1 day		1,380*	4,130	3,300	813
8 days		1,850	4,300	3,380	855
2 mos.		1,800	4,260	3,460	877

* Units of CCA activity per cc. of test solution. These values are all standardized against a common standard. (11)

In order to test these possibilities, four samples of viruses were prepared in 0.1 M pH 7 phosphate buffer, namely Lee virus at 2 mg. per cc., PR8 virus at 2 mg. per cc., Lee virus at 1 mg. per cc. plus PR8 virus at 1 mg. per cc., and finally, Lee virus at 1 mg. per cc. plus ultraviolet-inactivated PR8 virus at 1 mg. per cc. The ultraviolet-treated PR8 virus, prepared by Dr. W. M. Stanley (12), was devoid of infectivity or CCA activity. It was anticipated that this material might be as effective as active PR8 virus in exerting a protective influence on the Lee virus. At the same time, such a material possessed the advantage that it would not interfere, as would active PR8 virus, with measurements of changes in infectivity or CCA activity of the Lee virus. The results obtained in measurements of infectivity and CCA

activity are shown in Table VIII. A comparison of these data with those shown earlier in Tables II, IV, and VI reveals that both viruses exhibit stabilities of CCA activity and infectivity much greater in concentrated solution than in dilute solution. At the higher concentrations of 2 mg. per cc., no significant losses in either the infectivity or the CCA activity occurred during the test period of 2 months.[1] It can also be noted that the infectivity end-points shown in Table VIII are higher than those shown in previous tables. It is probable that this result is due to the fact that the purified virus preparations employed as starting materials in the present experiments were not subjected to the inactivating effect of the removal of electrolyte by centrifugation from distilled water which was necessary in earlier experiments when buffers other than phosphate at pH 7 were used. From the standpoint of the preparation of mixed vaccines from centrifugally concentrated strains of influenza virus (12), the results are of particular interest since they demonstrate not only conditions under which a maximum stability can be maintained but also that the strains are stable and apparently unchanged in the presence of one another.

DISCUSSION

The finding that influenza viruses are more stable in buffer media containing glycine and NaCl than in phosphate buffer alone is similar to the observation of Knight and Stanley that PR8 virus appears more stable in the presence of a mixture of arginine and phosphate than in phosphate buffer alone (13). In both instances the amino acids appear to exert a protective action on the virus. It can be pointed out in this connection that glycine has also been found to protect certain enzymes against inactivation (14, 15). The finding that the viruses were inactivated much more slowly at a protein concentration of 2 mg. per cc. than at 0.1 mg. per cc. is analogous to the observation of Price that the rate of thermal inactivation of tobacco mosaic virus was lower in undiluted than in diluted extracts of the virus (16).

The pH of maximum stability of the mouse infectivity of strains of influenza virus appears to agree quite closely with the pH of maximum stability of the CCA activity. Because of the fact that the quantitative test for CCA activity is much more sensitive than that for infectivity, no special significance can be attached to small differences in the apparent pH values for maximum stability in certain cases. The coincidence of optimum pH conditions for the two types of biological activity may be regarded as evidence that both activities arise from the same particle. Independent evidence obtained by means of

[1] Further tests were made after the present paper was submitted for publication. At the end of 4 months all virus samples showed 90 per cent or more loss in infectivity. The three samples containing Lee virus also showed considerable loss in CCA activity, whereas the sample containing only PR8 virus was unchanged. These findings confirm earlier observations that the Lee virus is less stable than the PR8 strain, and demonstrate that the presence of PR8 virus fails to protect the Lee virus against inactivation.

centrifuge studies has provided more conclusive proof for the identification of the two properties with a single physical unit (17, 18). The marked differences which have been observed between the rates of loss of CCA activity and of infectivity suggest, however, that the CCA activity is much less sensitive than the infectivity to alterations in virus structure. The observation that in alkaline solution virus infectivity is lost very rapidly while comparatively little change occurs in agglutination titer is similar to the finding of Hirst who noted that the treatment of the virus with heat or with formaldehyde caused a much greater decrease in infectivity than in agglutination titer (19).

SUMMARY

A study has been made of the pH stability of centrifugally purified strains of influenza virus with respect to the biological properties of mouse infectivity and chicken red blood cell agglutinating activity. Observations also were made on the importance of composition of buffer, temperature of storage, and concentration of virus protein to the stability of the virus.

When tested for stability at a protein concentration of 0.1 mg. per cc. in phosphate buffer, the infectivity of PR8 virus was found to be most stable at pH 6.5–7; the swine virus, at pH 7–7.9; and the Lee strain, at a pH of 7.9 or higher. The CCA activity of the PR8 virus in phosphate buffer was most stable at pH 7, that of the swine virus at pH 7–8, and that of the Lee virus at a pH greater than 9. Furthermore, the Lee virus was much less stable in dilute solution in phosphate buffer, even under optimum conditions of pH, than either the PR8 or swine strains.

The different strains of influenza virus were found to possess certain characteristics in common. They lost infectivity and CCA activity on the acid side of optimum pH conditions much more rapidly than on the alkaline side. Under suitable conditions of buffer and pH, the infectivity decreased while the CCA activity remained unchanged. In general, the rate of loss in infectivity was greater than the rate of loss in CCA activity.

When tests of stability were carried out at a protein concentration of 0.1 mg. per cc. in a composite phosphate-glycine-NaCl buffer, the virus strains showed less marked differences and possessed much higher stabilities of CCA activity and infectivity than when stored at the same concentration in phosphate buffer alone. Under the modified conditions, all three viruses possessed maximum stabilities of CCA activity and infectivity at pH 7–8 with the exception of the PR8 virus whose infectivity appeared more stable at pH 7 than at pH 8. In detailed experiments with the Lee virus, it was found that the infectivity and CCA activity of this strain at pH 7 and at a protein concentration of 0.1 mg. per cc. were maintained best in the composite phosphate-glycine-NaCl buffer, less well in a buffer containing glycine and NaCl, and least well in phosphate buffer alone.

In tests with PR8 virus, the activity was found to be much more stable at 4° C. than at 23° C.

When stored at a concentration of 2 mg. per cc. at 4° C. in phosphate buffer at pH 7, the PR8 and Lee strains were found to be much more stable than when stored at the concentration of 0.1 mg. per cc. At the higher concentration, no significant losses in either infectivity or CCA activity were observed over a period of 2 months.

BIBLIOGRAPHY

1. Andrewes, C. H., and Smith, W., *Brit. J. Exp. Path.*, 1937, **18,** 43.
2. Ostrovskaya, S. M., Czalkina, O. M., and Olechnowiteh, S. B., *Arch. sc. biol.*, 1938, **52,** 31.
3. Stock, C. C., and Francis, T., Jr., *J. Exp. Med.*, 1940, **71,** 661.
4. Hirst, G. K., *Science*, 1941, **94,** 22.
5. Miller, G. L., *J. Exp. Med.*, 1944, **79,** 173.
6. Stanley, W. M., *J. Exp. Med.*, 1944, **79,** 255.
7. Knight, C. A., *J. Exp. Med.*, 1944, **79,** 285.
8. Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80,** 531.
9. Clark, W. M., The determination of hydrogen ions, Baltimore, The Williams and Wilkins Co., 3rd edition, 1928.
10. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45,** 273.
11. Miller, G. L., and Stanley, W. M., *J. Exp. Med.*, 1944, **79,** 185.
12. Stanley, W. M., *J. Exp. Med.*, 1945, in press.
13. Knight, C. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **79,** 291.
14. Negelein, E., and Wulff, H. J., *Biochem. Z.*, 1937, **293,** 351.
15. Warburg, O., and Christian, W., *Biochem. Z.*, 1943, **314,** 149.
16. Price, W. C., *Arch. ges. Virusforsch.*, 1940, **1,** 373.
17. Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, **79,** 301.
18. Lauffer, M. A., and Miller, G. L., *J. Exp. Med.*, 1944, **80,** 521.
19. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.