THE STABILITY OF INFLUENZA VIRUS IN THE PRESENCE OF SALTS*

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The investigations of Andrewes and Smith, Ostrovskaya and colleagues, Stock and Francis, and of Eaton indicated that influenza virus in mouse lung suspensions is most stable between pH 6 and pH 8 (1–4). The more extensive studies of Miller, with purified egg-adapted virus, confirmed the earlier observations and established a zone of maximum stability at pH 6.5–7.0 (5). In all of these investigations, however, emphasis was placed upon the effect of hydrogen ion concentration without particular regard for the possible specific effects of the various salts involved. In much work with influenza virus, it has been customary to use physiological saline as the solvent, although there are no data available which would indicate that 0.85 per cent sodium chloride is the best medium in which to work with this virus. Because of the special effects found to be exerted by different solvents in studies on certain purified plant viruses (6), it appeared to be desirable to make similar studies on influenza virus. The present investigation was made to determine the relative stability of purified influenza virus on standing at 4°C. in various solvents at pH 7.

Materials and Methods

Preparation of Virus.—Allantoic fluid containing egg-adapted PR8 virus was obtained as previously described (7). After removing insoluble matter in a low-speed centrifuge, the allantoic fluid was spun in a high-speed centrifuge for about 2 hours (8). The supernatant liquid was discarded and the pellets were dissolved in the solvent to be tested. The solution obtained was centrifuged at low speed to remove insoluble matter. Protein analyses were made on the supernatant fluids by the Nessler method (9). All samples were diluted with the appropriate solvent until a final virus preparation containing about 10^{-4} gm. of protein per ml. was obtained. Samples were removed immediately and at intervals thereafter for activity tests in chick embryos and in mice. Dilutions for the latter tests were made in the same solvent in which the virus was stored, except as otherwise noted. Between tests, all solutions were stored at 4°C. The solvents employed included 0.001, 0.01, 0.1, and 1.0 M solutions of phosphate, about 0.1 M veronal and borate buffers, doubly distilled water, and physiological saline. The pH of the latter two solvents was adjusted to the neighborhood of

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7 by addition of 0.01 N sodium hydroxide. The phosphate and borate solutions were Sorenson's buffers (10), and the veronal buffer was prepared as described by Michaelis (11). All solutions were sterilized in an autoclave before use as solvents for the virus.

Tests for Virus Activity.—Tests were made in chick embryos and in mice as previously described (7).

TABLE I

The Effect of Salts on the Stability of 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

Salt	Initial pH	0 days	4 days	9 days	18 days			22 days			37 days*			Final pH
		10^{-8}‡	10^{-6}	10^{-8}	10^{-7}	10^{-8}	10^{-9}	10^{-8}	10^{-10}	10^{-11}	10^{-6}	10^{-8}	10^{-10}	
0.001 M phosphate....	7.2	+	+	+	+	+	+	+	0	0	+	+	0	6.7
0.01 M "	7.1	+	+	+	+	+	+	+	+	0	+	+	0	6.6
0.1 M "	7.1	+	+	+	+	+	+	+	+	+	+	+	+	6.8
1.0 M "	7.0	+	+	+	+	+	+	+	0	0	+	+	0	7.0
Distilled water.....	6.8	+	+	+	0	0	0				+	+	0	7.0
Saline.....	6.8	+	+	+	+	0	0				+	0	0	6.9
Veronal.....	6.9	+	+	+	+	+	+	+	+	+	+	+	0	7.0
Borate.....	7.0	+	+	+	+	+	+	+	+	+	+	+	+	7.2

* Test dilutions were made with the same salt solution in which the virus was stored for tests through 22 days, with the exception of M phosphate. The latter was lethal to chick embryos and was diluted therefore with 0.1 M phosphate for tests. All test dilutions at 37 days were made with 0.1 M phosphate.

‡ Figures for test dilutions indicate grams of protein per milliliter of test solution. Three eggs were used to test each dilution and each egg was injected with ⅓ ml. Allantoic fluids from the three eggs were pooled for testing. + indicates presence of virus.

EXPERIMENTAL

Stability of the Virus

The results of the tests which are summarized in Tables I and II demonstrate that in different solvents there may be an appreciable variation in the stability of influenza virus at the pH at which it is considered to be most stable. For example, it is apparent that considerable inactivation of the virus occurred in the distilled water and physiological saline solutions at the end of 18 days, despite the fact that the pH of these solutions was well within the range of maximum stability. Similarly, it is clear that at 22 days there was at least a hundred times more virus activity in the 0.01 M and 0.1 M phosphate solutions than there was in the 0.001 M and 1.0 M solutions. The 0.1 M phosphate, the veronal, and the borate buffers appeared to be almost equally effective in preserving virus activity under the conditions tested.

Inactivation and Reactivation of Virus

Perhaps the most striking result of the tests was the apparent increase in activity of the virus stored in distilled water when diluted with 0.1 M phosphate buffer. The preliminary data suggested that a 10- to 100-fold reactivation had been accomplished by diluting the virus with phosphate rather than with adjusted distilled water. To investigate this possibility, fresh virus was obtained by centrifuging infectious allantoic fluid in the customary manner. Some of the pellets were taken up in 0.1 M phosphate, some in adjusted distilled water, and some in ordinary distilled water. The pH of the first two solvents was about 7 and that of the ordinary distilled water was about 5.8. On the

TABLE II

The Effect of Salts on the Stability of the PR8 Strain of Influenza Virus on Standing at 4°
Mouse Tests

Salt	0 days	4 days	9 days	18 days
	10^{-6}*	10^{-6}	10^{-6}	10^{-5}
0.001 M phosphate	D_7,‡ D_7, D_8	D_7, D_8, 3	1, ±, 0	D_5, D_5, D_5
0.01 M "	D_4, D_6, D_7	D_6, D_7, 3	3, 2, 1	D_4, D_5, D_6
0.1 M "	D_6, D_7, D_7	D_6, D_6, D_6	2, 2, 1	D_4, D_7, D_8
1.0 M "	D_7, D_8, D_9	D_9, D_{10}, 2	1, 1, ±	±, ±, 0
Distilled water	D_7, D_7, D_7	D_6, D_7, 1	0, 0, 0	0, 0, 0
Saline	D_5, D_6, D_6	D_4, D_5, D_6	2, 1, 1	1, ±, ±
Veronal	D_7, D_8, D_8	D_5, D_5, D_5	1, 1, ±	D_6, D_6, 1
Borate	D_6, D_7, 3	D_5, D_6, D_6	2, 2, 1	D_6, 3, 2

* Figures for test dilutions indicate grams of protein per milliliter of test solution.

‡ The mice which died are listed as D, with a subscript denoting the day of death. The degree of pulmonary involvement in the animals surviving on the 10th day is indicated by numerals, with 4 = lung completely consolidated, 3 = ¾ consolidated, etc.

basis of protein analyses, each solution was diluted with the appropriate solvent to a protein concentration of approximately 10^{-4} gm. per ml. Samples were removed immediately for activity tests in chick embryos, and at the end of 9 days and 27 days for activity tests in both chick embryos and in mice. On the 27th day, dilutions for activity tests were made not only with the solvent in which the virus was stored but also with phosphate, borate, and veronal buffers. Between tests the solutions were stored at 4°C. In order to facilitate comparison of the results obtained in the chick embryo tests, 50 per cent infectivity end points were calculated by the method of Reed and Muench (12).

As shown in Table III, the virus in 0.1 M phosphate buffer was considerably more active than that in ordinary or adjusted distilled water and remained so throughout the tests. From the data for the tests carried out with chick embryos on the 27th day, it can be seen that the use of 0.1 M phosphate in-

creased the activity of the virus in ordinary distilled water by about 100 times and that of the virus in adjusted distilled water by about ten times. The 10-fold increase in activity is the minimum observed in two other repetitions of the experiment. On one occasion a 1000-fold increase was obtained. The veronal buffer exerted somewhat less reactivating influence than the phosphate and the borate appeared to be without effect. The results obtained in the mouse tests tended to corroborate those obtained with the chick embryos in that, at the dilution tested, no lesions were obtained except in the cases where 0.1 M phosphate was the diluent (last column, Table III).

TABLE III

Effect of Diluent on the Infectivity of Salt Solutions of the PR8 Strain of Influenza Virus

Original medium for virus	Solvent for test dilutions	50 per cent infectivity end points with 5 chick embryos per dilution			Infectivity in mice*	
		0 days	9 days	27 days	9 days 10^{-7}	27 days 10^{-6}
0.1 M phosphate.....	0.1 M phosphate	10^{-14}	$10^{-12.5}$	$10^{-10.5}$‡	D_9, 2, 1	D_5, D_7, D_7
Distilled water.....	Distilled water	$10^{-8.5}$	$10^{-8.5}$	$10^{-6.7}$	0, 0, 0	0, 0, 0
" "	0.1 M phosphate			$10^{-8.5}$		1, 1, 1
" "	Borate buffer			$10^{-6.7}$		0, 0, 0
" "	Veronal "			10^{-8}		0, 0, 0
Adjusted distilled water	Adjusted distilled water	$10^{-10.5}$	$10^{-10.5}$	10^{-7}	1, 1, 0	0, 0, 0
" "	0.1 M phosphate			10^{-8}		1, 1, 0
" "	Borate buffer			$10^{-6.5}$		0, 0, 0
" "	Veronal "			$10^{-7.5}$		0, 0, 0

* See footnotes, Table II.

‡ The correct value was at least that indicated, since tests were not made to determine the maximum infectivity.

Tests similar to but not so extensive as those just described were made with solutions of virus in physiological saline and in 0.1 M phosphate. The data presented in Table I indicated that the virus is considerably more stable in 0.1 M phosphate than in adjusted physiological saline. The more detailed experiment summarized in Table IV confirmed the earlier result. Although the virus solutions appeared to possess the same activity after 4 days, by the end of 6 weeks the virus in 0.1 M phosphate was 10,000 times as active as that in either adjusted or unadjusted saline. Furthermore, it was possible to effect a 10-fold reactivation of the saline-inactivated virus by diluting with 0.1 M phosphate.

In an attempt to determine whether aggregation and redispersion might be

involved in the reactivation of the virus by dilution with 0.1 M phosphate, turbidity and nitrogen measurements were made on virus solutions in various buffers. The results, while not conclusive, indicated that no great differences in turbidity and hence in the state of aggregation occurred in the various salt solutions. The opalescence of the solutions as measured in a photoelectric colorimeter decreased gradually as the nitrogen of the supernatant liquid decreased owing to denaturation and settling out of protein. However, with the possible exception of the M phosphate, none of the solutions showed a sufficiently large change in turbidity or in nitrogen content to account for a 10-fold difference in virus activity. Further studies are being made in an attempt to elucidate the mechanism of reactivation.

TABLE IV

Effect of Diluent on the Infectivity of Salt Solutions of the PR8 Strain of Influenza Virus

Original medium for virus	Solvent for test dilutions	50 per cent end points with 5 embryos per dilution				
		4 days	23 days	30 days	42 days	45 days
0.1 M phosphate.....	0.1 M phosphate	$10^{-12.5}$	$10^{-10.3}$*	$10^{-10.5}$	$10^{-10.5}$	$10^{-11.5}$
Physiological saline..	Physiological saline	$10^{-12.5}$	$10^{-8.5}$	$10^{-8.5}$	$10^{-6.5}$	$10^{-6.7}$
" "	0.1 M phosphate				$10^{-7.5}$	10^{-8}
Adjusted physiological saline.....	Adjusted physiological saline	$10^{-12.3}$	$10^{-9.5}$	$10^{-7.5}$	$10^{-6.5}$	

* See footnote ‡, Table III.

SUMMARY AND COMMENT

The stability of centrifugally purified PR8 influenza virus at pH 7 in 0.001, 0.01, 0.1, and 1.0 M phosphate buffers, in veronal and borate buffers, and in adjusted solutions of saline and distilled water was investigated. The results demonstrate that the stability of this virus can vary considerably at pH 7 depending upon the nature and concentration of the salts present. Borate, veronal, and phosphate buffers at a concentration of about 0.1 M showed almost equal ability to maintain virus activity over several weeks of time at 4°C. In many cases, it may prove inconvenient to use veronal buffer, however, because of the difficulty in determining protein concentration in its presence. The 0.1 M phosphate buffer has proved in tests not described here to be slightly more consistent in preserving virus activity than the borate and may, therefore, be considered slightly superior to the latter. It is apparent that unbuffered saline is a poor solvent for preserving virus activity regardless of pH. The activity of partially inactivated virus in distilled water and in saline solutions was increased ten to 1000 times by diluting such solutions with 0.1 M phosphate buffer. Some reactivation was also effected with veronal but not with borate buffers.

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