15525

Effect of Formalin in Increasing Heat Stability of Influenza Virus Hemagglutinin.*

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A study of the heat stability of influenza virus hemagglutinin was recently reported[1] in which strain and line variations were described. Additional findings of interest have been revealed in further studies employing the same technics. It is the purpose of this communication to report the observation of an increase in heat-stability of the hemagglutinin of influenza virus in the presence of low concentration of formalin, and to discuss certain extensions of the previous observation of strain differences as evidenced by the variable effect of formalin on several strains of virus.

Materials and Methods. The source of virus in these experiments, as before, was the allantoic fluid of chick embyros infected with the various strains. A constant temperature water bath at 61.5°C was used and the method for titrating hemagglutinin is the same as that employed before. The term formalin refers to a solution containing 40% of formaldehyde by volume.

Results. Formalin, in proper concentration, destroys the infectivity of influenza virus, but the immunizing property is retained.[2] Similarly, formalin will destroy infectivity without altering hemagglutinating capacity.[3] Since an excess of formalin will destroy not only infectivity, but other properties of the virus as well,[3,4] it was anticipated that in the presence of progressively increasing concentrations of the chemical there would occur a corresponding increase in rate of destruction of hemagglutinin at any given temperature. It was of interest to find that with 2 of the 3 strains originally studied, the stability of hemagglutinin at 61.5°C was considerably greater in the presence of 0.05% formalin than in the unformalinized control. Table I illustrates the effect observed when the concentration of formalin was varied in allantoic fluids containing the PR8 and Weiss strains of Type A virus and the Lee strain of Type B virus. From these data it is seen that the stability of the hemagglutinin of the PR8 and Weiss strains was enhanced in the presence of the lowest concentrations of formalin used. In contrast, the Lee strain of Type B virus showed a progressive reduction in stability of its hemagglutinin with increasing concentrations of formalin; even the smallest quantity had a deleterious effect. It is of interest that the 2 Type A strains differed with respect to the degree of difference between the untreated and the formalin-treated preparations and that the range of formalin concentration in which the effect was observed was greater for the Weiss than for the PR8 strain.

In view of these differences a number of other strains was examined to see if a type difference was involved. A group of 13 Type B strains was tested. Among these were the BON strain isolated in Doctor Burnet's laboratory in 1943,[5] 2 strains isolated from an outbreak of May 1945 and 10 strains isolated in this laboratory from the epidemic of 1945-46.[6] Some were tested with different quantities of formalin and others were tested only with the 0.05% concentration. In all 13 an increased stability of hemagglutinin was evident in the presence of the formalin. Table II illustrates the effect of different concentra-

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TABLE I.
Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (hr)	Concentrations of formalin, %				
		0	0.05	0.10	0.20	0.50
PR8	0	20,000+	20,000+	20,000+	20,000+	20,000+
(Type A)	.5	10,000	20,000+	20,000+	10,000	0
F-198	1	5,000	10,000+	10,000+	2,560	0
M-593	2.5	640+	5,000+	2,560	0	0
E-56	6	0	640+	0	0	0
	8	0	80+	0	0	0
WEISS	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type A)	.25	5,000+	10,000	5,000+	5,000+	2,560
F-3	.5	1,280+	5,000+	5,000+	5,000+	40
M-32	1	160+	5,000+	5,000+	5,000+	0
E-54	2	0	5,000+	5,000+	2,560+	0
	4	0	1,280	2,560+	640	0
	8	0	640	640	80	0
LEE	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type B)	1.5	10,000	10,000	160+	0	0
F-8	3	10,000	1,280	0	0	0
M-137	6	5,000+	0	0	0	0
E-102	9	5,000+	0	0	0	0
	16	1,280	0	0	0	0

TABLE II.
Rate of Destruction at 61.5°C of Hemagglutinin of the Amdall Strain* of Type B Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Time at 61.5°C (hr)	Concentrations of formalin, %						
	0	0.01	0.025	0.05	0.10	0.25	0.50
0	5000	5000	5000	5000	5000	5000	5000
.25	0	640+	2560	5000	5000	1280+	0
.5	0	320	1280+	2560+	5000	160	0
1	0	80	640	1280+	1280+	0	0
2	0	0	320	1280	1280	0	0

* In sixth egg-passage.

tions of formalin on one of the recently isolated Type B strains and Table III shows the effect of 0.05% formalin on several other strains as well. In terms of the effect of formalin in a concentration of 0.05%, it is seen that variations exist among the strains shown in Table III, all of which had been isolated from the same outbreak. It appears from these data that the Lee strain is exceptional among the Type B strains, thus far tested, with respect to the effect of the presence of formalin on the stability of the hemagglutinating component of the virus. In view of the variation in heat-stability of 3 different passage lines of the Lee strain reported previously,[1] it was of interest to find that all 3 lines behaved in the same exceptional way in the presence of formalin.

Table IV illustrates the observations made in tests of 3 Type A strains isolated in this laboratory, other than PR8 and Weiss. All 3 showed a slight enhancement of stability in the presence of formalin, but the effect was strikingly less marked than in the case of the Type B strains shown in Tables II and III. Moreover, a somewhat higher concentration of formalin was required to produce the slight effect evident in the Type A strains shown in Table IV.

In summary, these data show that in the

TABLE III.
Rate of Destruction of Hemagglutinin of 5 Strains of Type B Influenza Virus from the 1945-46 Epidemic. Comparison of Formalinized and Unformalinized Allantoic Fluids.

Time at 61.5°C (hr)	Amdall		Neubert		Sadowski		Mindell		Baker	
	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.
0	2560+	2560+	2560+	2560+	2560+	2560+	1280+	1280+	1280+	1280+
.25	0	—	0	—	0	—	0	—	*0	*160+
1	0	2560+	0	1280	0	640+	0	1280	*0	*40
2	0	2560	0	640	0	320	0	640+	*0	*0
4	0	1280	0	320	0	20+	0	320		
6	0	320	0	80	0	0	0	80		
8	0	20+	0	0	0	0	0	0		
10	0	0	0	0	0	0	0	0		

* Successive intervals were 5, 10, 15 minutes instead of time shown in first column.

TABLE IV.
Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Type A Influenza Virus in Allantoic Fluids Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (min)	Concentrations of formalin, %						
		0	.01	.025	.05	.10	.25	.50
Johnson	0	5000+	5000+	5000+	5000+	5000+	5000+	5000+
(1943)	15	20	20	40+	160+	160+	640+	40+
Type A	30	0	0	0	20+	160	320	0
F=0	60	0	0	0	0	20+	20+	0
M=0	120	0	0	0	0	0	0	0
E=9								
Scott	0	2560+	2560+	2560+	2560+	2560+	2560+	2560+
(1943)	5	0	0	0	20	40+	0	0
F=0	15	0	0	0	0	0	0	0
M=0								
E=7								
Baum	0	10,000	10,000	10,000	10,000	10,000	5000+	0
(1941)	5	640+	640+	2,560	2,560+	2,560+	0	0
Type A	15	0	0	0	40+	320+	0	0
F=1	30	0	0	0	0	0	0	0
M=10								
E=11								

presence of small concentrations of formalin the hemagglutinin of all but one of 19 strains of influenza virus tested is more stable when subjected to a temperature of 61.5°C and that the effect on different strains varies.

In an effort to determine the influence of time on the reaction between formalin and the hemagglutinating factor tests were made of heat stability at intervals after storage at 4°C. In mixtures of formalin and the Weiss or PR8 strains tested immediately after preparation or after storage for 2 days or 2 weeks, the inactivation curves were indistinguishable, provided the concentrations of formalin present were not great enough to cause destruction of hemagglutinin at the 4° storage temperature. In another experiment, in which formalin was added *after* the virus preparation had been heated for different periods of time, the hemagglutinating capacity was not restored; although in an aliquot the hemagglutinating capacity was retained when formalin had been added *prior* to heating for corresponding intervals. It would seem, therefore, that the presence of formalin delays the destructive effect of heat on hemagglutinin; however, the addition of formalin to a heated virus suspension does not reactivate the hemagglutinin.

Discussion. The observations reported in this communication are of interest with respect to several questions.

It has been noted with almost all strains of influenza virus examined that the heat-stability of the hemagglutinating property was enhanced in the presence of low concentrations of formalin. This is similar to the finding of Schmidt, Glenny, Ramon, and others who reported that the stability of diphtheria toxin to heat-denaturation was increased after treatment with formaldehyde for production of toxoid.[7] It is also known that formalinized pneumococci are more stable than unformalinized organisms[8] and that greater stability to the denaturing action of heat can be produced by formaldehyde treatment of proteins.[7] The exact mechanism of the formaldehyde effect, in which toxicity or infectivity is destroyed without impairing other antigenic properties, is unknown. Many hypotheses have been suggested.[7,9,10] The present observations support the speculation that in addition to blocking or destroying the chemical groupings necessary for toxicity or infectivity, some intramolecular rearrangement occurs producing an effect that might be described as "fixation" of other reactive groups.

A curious fact with respect to influenza virus is the variation observed in the behavior of the hemagglutinating factor of different strains in the presence of formalin. Lack of uniformity in the reaction of the various strains of virus with so simple a substance as formaldehyde suggests that the chemical groupings in the constitution of the individual strains differ widely, either quantitatively or qualitatively.

It is of further interest that differences in the formalin effect were observed among strains of virus isolated from different patients in the same geographical area during a recent epidemic of influenza B.[6] These same strains have been found to possess differences in serological[6] and physical[1] properties. Variations in virulence and serological differences among strains of influenza virus have been described previously[11] and the problems posed by strain variations have been discussed by Francis,[12] Andrewes,[13] Burnet,[14] and others. The fact that strains of virus from the same outbreak are intrinsically different indicates that variants manifested by differences in certain properties do occur very readily in nature. An agent, so labile in its genetic constitution as to vary in the course of multiplication in the same host or from host to host, may be expected to alter its biological behavior quite unpredictably. This characteristic may serve to explain, in part, the striking variability of influenza virus activity both clinically and epidemiologically.[15]

With respect to the more immediately practical question of influenza virus vaccines, the finding of an increased stability of the virus hemagglutinin after formalinization of all but one of the strains tested suggests that the problem of a useful inactivating agent had been solved. The exact relationship between hemagglutinating activity and immunizing activity is still under investigation. The trend of studies reported[16,17] as well as those in progress invites the speculation that the technics employed may furnish information of value with respect to the relative stability of the immunizing capacity of influenza virus vaccines prepared or treated in various ways.

Summary. The observation of an increase in heat-stability of the hemagglutinating property of influenza virus in the presence of low concentrations of formalin has been described. Differences in the effect of formalin on different strains has been reported and discussed.

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