STUDIES ON THE TOXICITY OF INFLUENZA VIRUSES

I. The Effect of Intracerebral Injection of Influenza Viruses*

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It is a common experience that the degree of prostration during and following an attack of influenza in man may be out of proportion to the clinical signs observed. The lesions are found to occur dominantly in the respiratory tract and, consequently, virus has been isolated thus far only from the nasal and pharyngeal secretions of patients and from the lung tissue of a few fatal cases. As uncomplicated influenza in man runs usually a mild course, it has not been possible to determine whether a spread of the virus from the respiratory tract to other organs takes place and whether such a spread might not be responsible for some of the general signs and symptoms associated with the disease.

In animals, likewise, the disease appears to be confined to the respiratory tract. Thus, virus has been recovered only from the lungs, tracheal exudate, and turbinates of infected pigs (1).[1] Only minute quantities of virus could be demonstrated under certain conditions in various other organs of mice (2, 3). A study of the infected guinea pig fetus revealed appreciable amounts of virus in a number of tissues other than those of the respiratory tract (4). However, the mere presence of virus in an organ does not necessarily imply that it has propagated there. Multiplication of the agents never has been demonstrated outside the air passages (5) except for two strains which could be adapted to the brain tissue of mice (6, 7) and of rabbits (8, 9).

Experiments conducted in this laboratory support the view that influenza viruses do not readily multiply outside the respiratory tract of mice. However, evidence has been found that these agents may exert a deleterious effect on various organs when injected by a non-infectious route, provided a sufficient quantity of the virus is administered. Thus, toxic manifestations have been demonstrated in this laboratory following intracerebral, intraperitoneal, and intravenous injection of certain influenza virus preparations (10, 11). The results of intracerebral inoculation have been confirmed by Hale and McKee (12). Evans and Rickard demonstrated toxic effects of influenza viruses in the absence of multiplication on the cornea of the rabbit (13). Similar toxic properties have been described previously in the case of *Rickettsiae* by

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[1] The references are listed at the end of the subsequent paper.

Gildemeister and Haagen (14) and by Bengtson, Topping, and Henderson (15) and in agents of the psittacosis-lymphogranuloma venereum group by Rake and Jones (16).

It is the aim of this series of papers to present studies on the toxic properties of the influenza viruses in greater detail. Possible applications of the phenomenon will be explored. The first communication will describe experiments in mice using the intracerebral route for the injection of the virus.

Materials and Methods

Virus.—The PR8, WS, F-12, F-99, and Weiss strains of influenza A, the Lee strain of influenza B, and the S-15 strain of swine influenza were used in these studies. Egg-adapted virus was obtained by inoculation of 10-day-old chick embryos by the allantoic route with 0.2 ml. of preparations of virus suitably diluted in brain-heart infusion broth. At the end of the indicated incubation period at 36–37°C. the eggs were chilled at 2°C. for 2 hours or at −15°C. for 30 minutes. The blood-free allantoic fluids were collected aseptically and used in the experiments or as starting material for the preparation of virus concentrates by various procedures.

Mouse-adapted virus which had been passed over extended periods of time by the intranasal route was likewise used. For these experiments lightly anesthetized Swiss white mice were inoculated intranasally with 0.05 ml. of a 0.1 or 10 per cent suspension of emulsified infected murine lungs. The inoculated mice were sacrificed 2 to 4 days after infection and the lungs emulsified in broth to form a 10 per cent suspension. After centrifugation at 2000 R.P.M. for 10 minutes the supernatant fluids were saved and used in the experiments.

Viral Titration in Chick Embryos.—Tenfold dilutions of the viral suspensions in broth were prepared and 4 to 6 10-day-old chick embryos were injected with 0.2 ml. of the various dilutions by the allantoic route. After incubation of the eggs at 36–37°C. for 48 to 72 hours, they were chilled and the individual allantoic fluids harvested and tested for agglutination of chicken red cells as an indication of infection. The number of doses (ID_{50}) per milliliter of a given preparation was calculated according to the method of Reed and Muench. Comparison of the titers after an incubation period of 48 hours with that obtained after 72 hours showed only a small increase in titer for the additional 24 hours of incubation. In most instances an incubation period of 48 hours was used.

Intracerebral Injection of Mice.—3- to 5-week-old Swiss white mice of 12 to 15 gm. weight were injected intracerebrally under light ether anesthesia with 0.03 ml. of the viral preparation. The mice were examined twice daily, suspended by their tails and twirled moderately in order to accelerate convulsions.

Hemagglutination Test.—Series of twofold dilutions of the viral preparations were made in saline solution (volume 1.0 ml.) and 1 ml. of a 2 per cent suspension of thrice washed chicken red cells was added by automatic pipette. The red cells were never older than 3 days. After incubation for 75 minutes at room temperature the degree of sedimentation was measured by means of a photoelectric cell according to the method of Hirst and Pickels (17). The final dilution of virus leaving between 0.63 and 0.5 per cent of the cells in suspension was considered the end point.

Neutralization of Influenza Virus.—The virus preparation was mixed with an equal amount of immune serum and the mixture was left at room temperature for about 2 hours. After this interval 0.03 ml. of the material was injected intracerebrally into mice, or 0.05 ml. intranasally under light ether anesthesia as a control of the potency of the serum. The sera were in part derived from mice hyperimmunized with influenza viruses propagated in mouse lung passages, in part from rabbits immunized against chick embryo tissue culture virus. Normal sera as well as sera against the heterologous type of virus served as controls.

Concentration of Virus.—For the concentration of influenza viruses methods were employed which have been described previously (18). For the adsorption of virus onto red cells and the elution therefrom the technic of Hirst (19) was employed.

EXPERIMENTAL

In the course of certain studies with the Lee strain of influenza B it had been found that bacteriologically sterile preparations of allantoic fluid induced death in mice within 48 hours after intracerebral inoculation. On examination of survivors it was observed that convulsions occurred when the mice were suspended by their tails and twirled somewhat. The mice showed first a marked tremor

TABLE I

Influence of Induced Convulsions on the Results of Intracerebral Inoculation of Influenza B Virus (Lee Strain)

Concentration of inoculum	Twirling* of mice	Results in mouse No. 1	2	3	4	5	6	7	8
32 times	−	D_2	D_2	D_2	D_2	D_2	D_2	D_2	D_2
	+	C_1	C_1	C_1	C_1	C_1	C_1	C_1	C_1
2 times	−	D_2	D_2	D_2	D_2	D_2	D_2	D_2	C_2‡
	+	C_1	C_1	C_1	C_1	C_1	C_1	D_2	C_2
Normal membrane particles	+	S	S	S	S	S	S	S	S

D_2 = mouse died on 2nd day after inoculation.

C_1 = mouse went into convulsion on 1st day after inoculation.

S = mouse survived 10 days without showing neurological signs.

* Mice were suspended twice daily by their tails and slightly twirled in order to induce convulsions.

‡ This mouse was observed in spontaneous convulsion.

and clonic convulsions which usually ended in tonic convulsions. Death frequently occurred at this time. Surviving mice were markedly spastic for a few minutes after the convulsion and refractory to renewed attempts to induce the reaction. A few mice survived repeated convulsions in the course of several days. Mice not undergoing the examination described were observed on occasion to die spontaneously with the same signs, death occurring during tonic convulsion. Table I records an experiment in which the effect of induced convulsions was compared with the results obtained spontaneously in mice previously inoculated by the cerebral route with one of two concentrations of influenza B virus. This agent had been concentrated from allantoic fluid by means of high speed centrifugation and the sedimented virus had been resuspended in a fraction of the original volume. The results of this experiment show that handling of the mice accelerated the reaction. In all further experi-

ments the mice were tested, therefore, twice daily as described, and the convulsions were recorded. Histological examination of the central nervous system showed the destruction of the ependymal lining of the ventricles. There was only slight evidence of meningeal involvement and the brain parenchyma was essentially unaffected. Control injections of sterile broth, normal allantoic fluid, or particulate components derived from normal chorio-allantoic membranes by differential centrifugation did not produce any neurological signs nor any comparable histological lesions.

Convulsions were observed in further tests not only with the Lee strain of influenza B, but also with a number of strains of influenza A virus. The infected allantoic fluids could be diluted, at most, four- to eightfold and still cause this reaction. Other preparations did not produce convulsions when

TABLE II

Neurological Signs Produced by Various Strains of Influenza Virus

Virus preparation*			Result of intracerebral injection									
Strain	Type	Hemagglutinin titer	Mouse No. 1	2	3	4	5	6	7	8	9	10
PR8	A	1:16,384	C_2	C_2	C_2	C_2	C_2	C_2	C_2	C_2	C_2	C_2
WS	A	1:16,384	D_2	D_2	D_2	D_2	D_2	C_2	C_2	C_2	C_2	C_2
F-12	A	1:1,024	D_2	D_2	D_2	C_2	C_2	C_2	C_2	C_2	C_2	D_2
F-99	A	1:1,024	D_1	D_2	D_2	D_2	D_2	C_2	C_2	C_2	C_2	D_2
Weiss	A	1:16,384	D_2	C_2	C_2	C_2	C_2	C_2	C_2	C_2	C_2	S
Lee	B	1:4,096	D_1	D_2	D_2	D_2	C_2	C_2	C_2	C_2	C_2	C_2
S-15	Swine	1:16,384	D_1	D_2	D_2	D_2	D_2	D_2	D_2	D_2	C_2	C_2

* Concentrated 32 times from allantoic fluid by high speed centrifugation.

injected undiluted and only after concentration of the virus by means to be discussed later was this reaction observed. Table II lists the various strains of virus used and the results obtained with centrifugally concentrated preparations of the virus upon intracerebral inoculation. As long as sufficient quantities of virus were injected all mice died either between examinations or upon induced convulsions. Although the hemagglutination titers of the preparations varied markedly according to the strain no corresponding differences were noted under the conditions of the experiment in regard to the production of convulsions and death. No quantitative comparisons between the activities of various strains have been made as yet.

Neurological signs developed in mice upon the intracerebral injection of influenza viruses regardless of whether the agent was propagated in the allantoic cavity of the chick embryo or in the respiratory tract of mice. Strains of virus which had been passed continuously in mice by injection of infected mouse lungs by the intranasal route were used in several experiments and some of the results

are summarized in Table III. Intracerebral injection of normal mouse lung suspensions was uniformly without effect.

The fact that all strains of influenza virus tested gave similar results excluded the possibility of accidental contamination of preparations of influenza virus with a neurotropic agent. The possibility of activating a latent virus in the mice had to be considered, although control materials, as mentioned, did not produce any convulsions. The short incubation period seemed to exclude the virus of lymphocytic choriomeningitis. To assure against such a possibility preparations of influenza virus were tested in four differing strains of mice.

TABLE III

Neurological Signs Following Intracerebral Injection of Mouse-Adapted Influenza Virus

Preparation of virus			Route of injection	Results in mouse No.					
Strain	Dilution of inoculum*	Incubation period		1	2	3	4	5	6
PR8	Undiluted	72 hrs.	Intranasal	D_2	D_3	D_3	D_4	D_7	
			Intracerebral	C_1	C_1	C_1	C_3	C_5	D_9
F-12	Undiluted	72 hrs.	Intranasal	D_3	D_3	D_3	D_3	D_4	
			Intracerebral	C_1	C_2	C_3	D_4	D_{10}	S
PR8	Undiluted	48 hrs.	Intracerebral	C_1	C_1	C_2	C_2	C_2	C_4
	10^{-2}	48 "	"	D_1	D_3	D_3	D_5		
		72 "	"	C_2	C_2	C_2	D_3	D_6	
		96 "	"	D_3	D_3	D_6	S	S	
Normal mouse lung			Intracerebral	S	S	S	S	S	S

* 10 per cent suspension of mouse lung.

These were: (A) a strain of Swiss mice obtained from the dealer regularly supplying this laboratory; (B) a strain of Swiss mice originally obtained from Dr. L. T. Webster and maintained in a colony at The Children's Hospital of Philadelphia under conditions of strict isolation; (C) and (D) a white and a brown strain of mice kindly supplied by Dr. O. H. Meyerhof who had obtained them from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

As can be seen in Table IV, all strains reacted with convulsions to the injection of the virus preparations. Although the Bar Harbor strains reacted more readily than the other two strains of mice, no definite statement as to greater susceptibility can be made at present since no quantitative comparisons have been attempted.

In addition to these various strains of mice the effect of intracerebral injection of influenza viruses was tested in some other species of animals, *i.e.*, hamsters

(0.1 ml.), guinea pigs (0.1 ml.), and white rats (0.06 ml.). All animals showed some response to the injection. The reaction of the rats was similar to that observed in the mice, in that the same type of clonic-tonic convulsions was induced upon suspension of the animals by their tails. The other two species, however, did not show convulsions when suspended by their hind legs. However, both the hamsters and the guinea pigs became lethargic 24 hours after the injection and had difficulty in maintaining their equilibrium. In addition, the guinea pigs revealed a spastic gait for several days. All animals in these experiments recovered.

TABLE IV

Neurological Signs Produced in Different Strains of Mice by the Intracerebral Injection of Influenza Virus

Strain of mice		Preparation of virus	Result of intracerebral injection					
Desig-nation	Color		Mouse No. 1	2	3	4	5	6
A	White	PR8 allantoic fluid (1)	D_1	D_6	S	S	S	S
B	"	" " " (1)	C_2	C_4	C_5	S	S	S
A	White	PR8 allantoic fluid (2)	C_2	C_2	C_3	C_4	C_5	S
B	"	" " " (2)	C_1	C_1	C_2	C_3	S	S
A	White	PR8 allantoic fluid (3)	D_1	D_1	C_2	C_2	C_2	S
B	"	" " " (3)	C_1	C_2	C_2	C_2	C_2	S
A	White	PR8 32 times concentrated	C_2	C_2	C_2	C_2	C_2	C_2
C	Brown	" 32 " "	C_1	C_1	C_1	C_1	C_1	C_2
D	White	" 32 " "	C_1	C_1	C_2	C_2	C_2	C_2
A	White	Lee 32 times concentrated	C_2	C_2	C_2	C_2	C_2	C_2
C	Brown	" 32 " "	D_1	C_1	C_1	C_1	C_1	C_2
D	White	" 32 " "	C_1	C_1	C_1	C_1	C_1	C_2

Attempts at Propagation of the Viruses by the Cerebral Route

From the foregoing experiments it seemed very likely that the agents involved in the reaction were the influenza viruses. However, all attempts to pass the agents from brain to brain failed under a variety of conditions with 6 different strains of influenza virus. Thus, mice developing convulsions on the 2nd, 3rd, or 5th day after intracerebral inoculation of infected allantoic fluid, were sacrificed and their brains removed for passage. In one instance the brain of a mouse dying on the 7th day was used. On passage of suspensions of these brains to mice by the intracerebral route no reactions were noted although subculture in chick embryos usually demonstrated the presence of residual amounts

of influenza virus. On further passage at 3-day intervals the virus was lost in all cases. Some of these data are recorded in Table V.

In another series, twelve transfers from brain to brain were made with the PR8, WS, Lee, and S-15 strains of virus with either 10 or 0.1 per cent suspensions of brain. Half of the mice

TABLE V

Attempts at Passage of the Toxic Agent by the Intracerebral Route

Virus	Intracerebral passage No.	Result of intracerebral injection	Result of subinoculation of chick embryos
PR8	1	C_2 C_2 C_2 C_2 C_2	4/4*
	2	D_3 K_3‡ K_3 K_3 K_3 K_3	1/6
	3	K_3 K_3 K_3 K_3 K_3 K_3	0/5
	4	S_{10} S_{10} S_{10} S_{10} S_{10} S_{10}	
WS	1	C_2 C_2 C_2 C_2 C_2	3/3
	2	K_3 K_3 K_3 K_3 K_3 K_3	0/6
	3	K_3 K_3 K_3 K_3 K_3 K_3	0/5
	4	S_{10} S_{10} S_{10} S_{10} S_{10} S_{10}	
F-99	1	C_2 C_2 C_2 C_2	4/4
	2	K_3 K_3 K_3 K_3 K_3 K_3	5/5
	3	K_3 K_3 K_3 K_3 K_3 K_3	0/5
	4	S_{10} S_{10} S_{10} S_{10} S_{10} S_{10}	
Lee	1	C_3 C_3 C_3 C_3	0/8
	2	K_3 K_3 K_3 K_3 K_3 K_3	0/6
	3	K_3 K_3 K_3 K_3 K_3 K_3	0/5
	4	S_{10} S_{10} S_{10} S_{10} S_{10} S_{10}	
S-15	1	C_2 C_2 C_2	4/4
	2	K_3 K_3 K_3 K_3 K_3 K_3	0/6
	3	K_3 K_3 K_3 K_3 K_3 K_3	0/5
	4	S_{10} S_{10} S_{10} S_{10} S_{10} S_{10}	

* 4/4 = four out of four embryos developed hemagglutinins.

‡ K_3 = mouse killed on 3rd day for passage of the brain.

were used for passage, the other half were saved in order to see whether or not neurological signs would develop at a later date. Again the results were negative. No virus could be demonstrated in the second- or third-passage brains by subinoculation of chick embryos. No neurological signs developed in any of the mice with one exception, nor did the mice develop immunity to the strain used for the attempted passage. In the exceptional case, *i.e.*, the sixth passage of the PR8 strain using the 10 per cent suspension of brain, a virus was activated which was identified serologically as a strain of lymphocytic choriomeningitis (LCM). This virus was neutralized by guinea pig anti-LCM serum[2] and not by anti-PR8 serum, and conversely,

[2] Kindly supplied by Dr. B. Hampil.

the toxic effect of allantoic fluid infected with PR8 virus was not neutralized by the anti-LCM but by the anti-PR8 serum.

These data indicated the absence of multiplication of influenza virus in the brain under the conditions of the experiments. Further evidence was supplied by studying the virus content of the brains of mice at varying time intervals after injection.

TABLE VI

Failure of Propagation of the Toxic Agent in Brain Tissue

Virus (allantoic fluid)				Virus titer in brains (ID_{50}/ml. for chick embryos)						
				Days after injection						
Strain	Type	Dilution	ID_{50}/ml. for chick embryos	0	1	2	3	4	7	10
Lee	B	Undiluted	$10^{9.7}$			$10^{3.7}$				
"	B	Undiluted	$10^{10.4}$	$10^{5.2}$		$10^{2.7}$* $10^{2.2}$‡				
"	B	Undiluted	$10^{9.7}$	$10^{6.0}$	$10^{3.0}$	$10^{2.7}$* $10^{3.0}$‡	$10^{1.4}$			
PR8	A	Undiluted	$10^{10.0}$	$10^{5.9}$	$10^{3.7}$	$10^{3.4}$				
WS	A	Undiluted	$10^{10.0}$	$10^{5.7}$	$10^{4.2}$	$10^{4.5}$				
S-15	Swine	Undiluted	$10^{10.2}$	$10^{4.7}$	$10^{3.5}$	$10^{3.0}$				
PR8	A	1:2	$10^{9.7}$	$10^{4.2}$		$<10^{2.5}$		$<10^{1.7}$	$<10^{1.7}$	$<10^{1.7}$
WS	A	1:2	$10^{9.0}$	$10^{2.2}$		$10^{1.7}$			$<10^{1.7}$	
Lee	B	1:2	$10^{9.0}$	$<10^{3.0}$		$<10^{1.7}$		$<10^{1.7}$	$<10^{1.7}$	$<10^{1.7}$
PR8	A	1:100	$10^{7.7}$			$10^{1.0}$		$<10^{0.7}$	$<10^{0.7}$	$<10^{0.7}$
WS	A	1:100	$10^{7.9}$	$10^{1.0}$		$10^{1.0}$		$<10^{0.7}$	$<10^{0.7}$	$<10^{0.7}$
Lee	B	1:100	$10^{7.0}$	$10^{1.0}$		$<10^{0.7}$		$<10^{0.7}$	$<10^{0.7}$	$<10^{0.7}$
S-15	Swine	1:100	$10^{8.2}$	$10^{1.7}$		$<10^{0.7}$		$<10^{0.7}$	$<10^{0.7}$	$<10^{0.7}$

* Brains from mice with convulsions.

‡ Brains from mice with no convulsions.

A number of mice were injected intracerebrally with influenza virus and groups of four animals were sacrificed either immediately after the injection or 1 to 10 days thereafter. The brains were removed aseptically and stored at −15°C. Emulsions were prepared from the organs just prior to the titrations of the virus content in chick embryos. Samples of allantoic fluid used for the original injection were likewise stored at −15°C. and titrated in eggs at the same time as the brain suspensions. The 50 per cent infectivity end points were calculated.

As can be seen in Table VI, 0.03 ml. of the preparations of virus containing between 1 million and 1 billion ID_{50} for chick embryos were injected intracerebrally in the various experiments but only a fraction of the amount could be demonstrated on harvest immediately after injection. The amount of virus

demonstrable in brain tissue rapidly decreased in 24 hours and more slowly from then on, until virus could no longer be recovered in 4 days or thereafter. It should be pointed out that the results of the titrations were similar regardless of whether the brains were removed from mice showing convulsions or from animals without neurological signs. With the more dilute inocula the virus seemed to disappear at a faster rate as compared with the results following injection of more concentrated preparations.

Relation of the Neurotoxic Agent to the Hemagglutinating and Infecting Properties

The experiments reported in the previous section indicated that influenza viruses usually do not multiply in the central nervous system. It appeared likely, therefore, that the effect of the intracerebral injection was caused by toxic activities of the viral preparations. Toxic properties should, in part, depend

TABLE VII

Relations of the Hemagglutinin Titer to the Incidence of Convulsions

Hemagglutinin titer	Virus			
	PR8		Lee	
	No. of mice	Per cent with convulsions	No. of mice	Per cent with convulsions
1:16 or less	6	0	30	0
1:32 to 1:64	18	0	64	19
1:128 to 1:256	84	41	124	61
1:512 to 1:1,024	100	70	78	71
1:2,048 or more	70	99	38	100

on the quantity of virus present in a given preparation. An analysis of the results of the various tests in regard to hemagglutinin titer and toxicity is shown in Table VII. It can be seen that those preparations of the PR8 and Lee strains which revealed the lower hemagglutinin titers were also less effective when injected intracerebrally. With increasingly higher titers the incidence of convulsions increased correspondingly.

In order to study further the relationships between influenza virus, as measured by hemagglutination and infectivity on the one hand, and the toxic activity on the other, virus was propagated in the allantoic cavity under a variety of conditions.

The dilution of the inoculum was varied as well as the period of incubation of the eggs, while all other conditions were kept constant. The allantoic fluids were harvested in the usual way and aliquots were injected immediately thereafter into mice. Agglutination tests were performed on the day of harvest and usually again when all the preparations of a given test had been collected. Titration of the virus activity in the chick embryos was done in a few tests only. All fluids were kept frozen at −15°C. until these tests were possible.

Table VIII summarizes several such experiments. It can be seen that allantoic fluids derived from dilute seeds usually exhibited greater toxicity than those obtained from more concentrated inocula. This behavior parallels the findings reported previously (20) in regard to infectivity. The hemagglutinin titer in some cases has reached its peak before the toxicity has been fully developed. On the other hand, the intracerebral toxicity disappeared again on prolonged incubation of the eggs, whereas the hemagglutinin titer was not affected. In the few instances in which the tests were combined with determinations of infectivity for chick embryos, no clear-cut relationship between toxicity and infectivity was apparent. Similar experiments employing the intravenous and intra-abdominal routes of injection are discussed in the subsequent paper. These data have shown that the toxicity reached its peak only after the infectivity had attained maximal titer.

Properties of the Toxic Agent

Although the experiments summarized above do not leave doubt as to the origin of the toxic principle it remained to be seen whether the property was inherent in the virus particle or a soluble substance derived either from the virus or from the infected host cell. This possibility was studied by a number of technics. By none of these was it found possible to separate the toxic activity from the virus particle. It was not dialyzable through cellophane. It sedimented in the high speed centrifuge in gravitational fields which also precipitated the infective and hemagglutinating properties. After 20 minutes at 20,000 R.P.M. the toxic agent was removed from the allantoic fluid and it could be recovered, although with some loss of activity, in the sediment together with the hemagglutinins. An experiment of this kind is summarized in Table IX. This table shows in addition another experiment with an allantoic fluid which did not contain enough of the toxic principle in suspension to cause neurological reactions in mice. However, on sedimentation of the virus and resuspension of the sediment in a small volume of saline solution the toxic agent was concentrated sufficiently to produce convulsions upon intracerebral injection. The small component obtained from allantoic fluids by centrifugation at 30,000 R.P.M. which possesses a sedimentation constant of about 30 S (21) in contrast to the sedimentation constant of about 600 S for the virus, was without effect upon intracerebral administration.

Other means of concentrating influenza virus, likewise concentrated the toxic activity of the preparations. Thus, experiments on adsorption of the virus onto chicken red cells at 0°C. and subsequent elution therefrom at 37°C. into one-tenth of the original volume of saline solution showed that the absorbed allantoic fluid lost most of the infectivity and no longer caused convulsions, whereas the eluate contained a high titer of virus and was toxic upon intracerebral injection. Precipitation of the virus by protamine (22) and re-

TABLE VIII

Optimal Production of the Neurotoxic Property

Strain of virus	Test	Dilution of inoculum													
		10^{-2}				10^{-4}				10^{-6}				10^{-8}	
		Time of incubation, *hrs.*													
		24	48	72	96	24	48	72	96	24	48	72	96	24	48
Lee	Hemagglutinin titer	—	1:64	1:32	1:24	—	1:224	1:192	1:192	—	1:384	1:256	1:384	—	—
	Toxicity	—	0/10*	3/10	1/10	—	8/10	9/10	0/10	—	10/10	10/10	6/10	—	—
PR8	Hemagglutinin titer	1:384	1:256	1:256	—	1:1,536	1:1,024	1:1,024	1:384	1:512	1:2,048	1:1,024	1:1,024	—	—
	Toxicity	2/6	3/6	1/6	—	5/6	6/6	3/6	1/6	5/6	6/6	2/6	2/6	—	—
F-12	Hemagglutinin titer	1:32	1:32	—	—	1:64	1:64	—	—	1:64	1:64	—	—	—	—
	Toxicity	3/6	1/6	—	—	6/6	1/6	—	—	3/6	0/6	—	—	—	—
F-99	ID_{50}/ml.	$10^{9.2}$	$10^{9.0}$	—	—	$10^{9.2}$	$10^{9.5}$	—	—	$10^{9.5}$	$10^{10.2}$	—	—	—	—
	Hemagglutinin titer	1:16	1:24	—	—	1:250	1:160	—	—	1:32	1:256	—	—	—	—
	Toxicity	0/6	0/6	—	—	3/6	0/6	—	—	0/6	5/6				
Lee	ID_{50}/ml.	—	$10^{6.8}$	—	—	—	$10^{9.7}$	—	—	—	$10^{9.2}$	—	—	—	$10^{9.9}$
	Hemagglutinin titer	—	1:512	—	—	—	1:1,024	—	—	—	1:1,280	—	—	—	1:220
	Toxicity	—	0/10	—	—	—	1/10	—	—	—	6/8	—	—	—	0/10
S-15	Hemagglutinin titer	1:128	1:128	—	—	1:128	1:256	—	—	1:2	1:256	—	—	—	—
	Toxicity	4/6	3/6	—	—	2/6	6/6	—	—	1/6	5/6	—	—	—	—

— = not tested.

* 0/10 = none of 10 mice showed convulsions.

suspension of the precipitate in a small volume of saline solution gave similar results.

Further attempts to differentiate between the toxic and infective properties were made by employing various technics of inactivation. Irradiation of viral preparations by ultraviolet light for periods of time which are known to destroy completely the infective property (23, 24) also destroyed the ability to elicit convulsions. Heating at 56°C. for 30 minutes may leave some active virus (20). Intracerebral inoculation of such preparations caused the death of two mice but no convulsions were noted. In the light of experiments recorded in the second paper of this series it is possible that some difference in the stability of the two

TABLE IX

Sedimentation of the Toxic Agent by High Speed Centrifugation

Preparation	Dilution	Virus													
		PR8							Lee						
		Hemagglutination titer	Result of intracerebral injection						Hemagglutination titer	Result of intracerebral injection					
			1	2	3	4	5	6		1	2	3	4	5	6	7
Allantoic fluid	Undiluted	1:768	C_5	D_6	S	S	S	S	1:256	S	S	S	S	S	S	S
Concentrate 100 times	"	1:25,000	D_1	D_1	D_1	D_1	C_2	C_2	1:16,000	D_2	D_2	D_2	D_2	D_2	D_2	D_4
Allantoic fluid	Undiluted	1:384	D_2	C_2	C_2	C_2	C_2	S	1:256	C_2	C_2	C_2	S	S	S	
	1:2		C_2	C_2	C_2	D_5	D_5	S		C_3	C_5	S	S	S	S	
	1:4		C_2	C_2	S	S	S	S		D_7	S	S	S	S	S	S
20 min. 20,000 R.P.M. supernate	Undiluted	1:16	S	S	S	S	S	S	1:6	S	S	S	S	S	S	
20 min. 20,000 R.P.M. sediment 4 times concentrated	"	1:1,024	C_1	C_1	C_2	C_2	C_2	C_4	1:768	C_2	C_2	C_2	C_2	S	S	
	1:2		C_2	C_2	C_2	C_2	C_2	C_4		C_2	C_2	C_2	C_2	S	S	
	1:4		C_2	C_2	C_2	C_2	C_2	C_2		D_2	C_2	C_2	S	S	S	
	1:8		C_6	S	S	S	S	S		D_3	D_3	S	S	S	S	
	1:16		S	S	S	S	S	S		D_3	S	S	S	S	S	

properties may be found on using shorter periods of inactivation, the infectivity being more susceptible to the effect of inactivating agents.

The question of stability of the toxic property under various conditions of storage has not been studied to any extent with regard to intracerebral injection. It is likely that the data presented in the subsequent paper on intraperitoneal and intravenous injections may be applicable to the intracerebral route since it is felt that both types of toxic activity are based on the same virus constituent. The few tests performed indicated that the toxic property may be stable for at least 8 days in the refrigerator in the case of allantoic fluids infected with influenza virus. After 7 weeks at 4°C. no convulsions could be elicited. However, concentrated viral preparations prepared by high speed centrifugation were of greater stability, and after 7 weeks about 50 per cent of the activity was still left.

TABLE X

Specific Neutralization of Toxic Principle by Immune Sera

Serum		Test virus				
vs.	Source	PR8	WS	LEE	S-15	Saline
PR8....	Rabbit	S S S S S S	D_1 D_2 D_2 C_2 C_2 S	D_1 C_2 C_2 D_2 D_2 C_2	D_1 C_1 C_1 C_1 C_1 C_2	S S S S S
WS.....	Mouse	C_5 C_5 S S S S	S S S S S S	C_2 C_2 C_2 C_2 C_2 C_2	C_1 C_1 D_2 D_2 D_2 D_3	S S S S S
Lee.....	"	C_1 C_1 C_1 C_1 C_1 C_1	C_1 C_1 C_1 C_2 C_2 C_2	S S S S S S	D_1 C_1 C_1 C_1 D_2 S	S S S S S
S-15....	Rabbit	C_1 C_2 C_2 C_2 D_3 S	C_1 D_2 C_2 C_2 C_2 S	C_2 D_2 D_2 D_2 C_2 C_2	D_1 D_5 S S S S	S S S S S
Saline...	—	C_1 C_1 C_2 C_2 C_2 C_5	C_1 C_1 C_2 C_2 C_2 S	C_2 C_2 C_2 C_2	C_1 C_2 C_2 D_5 S S	

Immunological Data

The experimental data thus far have shown that certain preparations of influenza virus may produce convulsions and death in mice upon intracerebral injection and that this property is closely linked to the virus particle. In the following experiments it will be shown that the property is type-specific and to some extent also strain-specific. Similar serological behavior has been well established in the neutralization of the infective and hemagglutinating properties. Cross-neutralization tests with immune sera derived from various experimental animals gave the results shown in Table X. Anti-influenza A sera protected against the toxic effects of influenza A strains but not against those caused by influenza B and swine virus. Anti-influenza B serum prevented convulsions only in the case of influenza B virus, and the serum against swine influenza virus, which was of low potency, gave some protection against the homologous virus and not against the heterologous strains. Strain differences are apparent from the results with PR8 and WS strains of influenza A with their corresponding immune sera. Only the homologous virus was completely neutralized.

Attempts to immunize mice actively against the toxic effects have not yielded uniform or clear-cut results. The following considerations may explain this difficulty: Only relatively large amounts of influenza virus have produced convulsions with regularity. On the other hand, experience gained with several neurotropic viruses has shown that the intracerebral challenge of vaccinated mice constitutes a much more severe test than administration of virus by other routes. It is possible, therefore, that the procedures employed for the immunization in the present study were frequently insufficient to develop antibody levels effective against virus injected by the intracerebral route, whereas they sufficed to confer solid protection against intranasal challenge. Although the tests conducted have given slight evidence of specific protection, the experiments were discontinued in favor of studies involving toxic effects of intra-abdominal or intravenous injection of virus. In these experiments protective effects of active immunization were clearly demonstrated as will be shown in the following paper.

DISCUSSION

The toxicity of influenza virus demonstrated by intracerebral administration will be discussed in the following publication, together with the results of intravenous and intra-abdominal injection of these agents. It is felt that the toxic properties demonstrated in the two papers are probably based on the same component of the virus.

One point, however, requires discussion here: an apparent discrepancy between the observations of Stuart-Harris (6) and Francis and Moore (7), on the one hand, and the present study on the other. Whereas the earlier investiga-

tors reported adaptation of certain strains of influenza virus to brain tissue, no such results were obtained in this laboratory. However, differences in the technics employed should be noted. In the successful cases the neurotropic tendencies developed as a result of numerous brain to brain passages after prolonged incubation periods. In the present study, neurological signs were noted within 5 days on first passage by the intracerebral route, but no lesions developed on further transfers which were made at short intervals. Therefore, the results presented in this paper do not contradict the reports in the literature but serve to emphasize the difference in the two types of reaction reported: infection and multiplication on the one hand, as against toxicity on the other. In the earlier reports (6, 7) the virus became gradually adapted to brain tissue and multiplied sufficiently to produce neurological signs, whereas in the present experiments, injections of large quantities of virus resulted in toxic reactions on first passage without adaptation of the agents to the central nervous system.

SUMMARY

Intracerebral injection of preparations of influenza viruses into mice led to tonic and clonic convulsions and death in tetanus, usually within 24 to 72 hours. Histological examination revealed the destruction of the ependymal lining of the ventricles as the dominant finding.

These reactions were obtained in four different strains of mice as well as in rats, guinea pigs, and hamsters. They were observed in mice after injection of mouse and egg-adapted virus, and all strains of virus tested gave these responses as long as sufficient quantities of the agents were injected. Control materials, such as normal allantoic fluids, particulate components of normal chorio-allantoic membranes, and suspensions of normal murine lungs, gave uniformly negative results. Activation of a latent virus as well as the inadvertent admixture of a neurotropic agent to the influenza cultures was excluded.

Propagation of the influenza viruses in the central nervous system could not be demonstrated and, in fact, the agents were no longer demonstrable in 4 days. It was concluded that the observed neurological reactions were the result of toxic activities rather than of virus propagation.

Comparison between the infectivity, hemagglutinating capacity, and the toxic activity showed that preparations with the higher titers of virus and hemagglutinin were also the more toxic ones.

The toxic agents could not be separated from the infectious particles by the use of such technics as differential high speed centrifugation, adsorption onto and elution from chicken red cells, and precipitation by protamine.

The toxic effect of influenza A virus preparations was specifically neutralized by anti-influenza A and not by anti-influenza B serum, and conversely. In addition, antigenic differences were noted between two strains of influenza A virus by this method of testing.