STUDIES ON THE TOXICITY OF INFLUENZA VIRUSES

II. The Effect of Intra-Abdominal and Intravenous Injection of Influenza Viruses*

By WERNER HENLE, M.D., and GERTRUDE HENLE, M.D.

(From The Children's Hospital of Philadelphia (Department of Pediatrics, School of Medicine, University of Pennsylvania), Philadelphia)

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In the preceding paper experiments were described on the effect of intracerebral injection of influenza virus preparations. Convulsions were noted in mice within 24 to 72 hours and occasionally later, without demonstrable propagation of the agents in the central nervous system. It was concluded that the influenza viruses may exert toxic effects on an organ which usually does not support the multiplication of these agents. In the course of further studies it became apparent that toxic properties could be demonstrated also by using other routes of administration. Thus, intra-abdominal and intravenous injection of potent viral suspensions caused death of mice within 8 to 96 hours, with lesions resembling in many respects those described by Rake and Jones (16) for viruses of the psittacosis–lymphogranuloma venereum group. A preliminary note on these experiments has been published (11). The present paper describes the experiments in detail and extends the information already reported.

Methods and Materials

Viruses.—The PR8, WS, Melbourne, F-12, F-99, and Weiss strains of influenza A, the Lee and ES strains of influenza B, and the S-15 strain of swine influenza virus were used. The technics employed for the preparation of infectious allantoic fluids, the methods for the titration of the virus activity by injection of chick embryos and by the hemagglutination tests, and the methods for the concentration of the virus and the neutralization tests have been described in the preceding paper.

Intravenous and Intraperitoneal Injections.—The virus preparations or dilutions thereof in buffered saline solution were injected by means of a 27 gauge needle intravenously into one of the tail veins of mice weighing 12 to 15 gm. The animals were placed into a small cardboard box with an attached folding lid and only the tail remained outside. The lid was pressed down onto the base of the tail with the middle finger, while the tail was held between thumb and index finger and stretched. This procedure fulfilled the function of a tourniquet and the vessels were usually sufficiently extended so that application of heat was not a necessary requirement.[1] However, for recent larger experiments the mice were placed just before the

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[1] This technic has been suggested by Dr. T. N. Harris.

injection into a wooden box heated by a 100 watt electric bulb which was separated from the mice by a wire screen. Usually 1 ml. of virus preparation was injected, which caused no noticeable distress to the mice.

For the intra-abdominal injection, a 25 gauge hypodermic needle was slipped between skin and muscle of the left lower quadrant of the abdominal wall for about 1 cm. before penetrating into the peritoneal cavity. This prevented backflow of the virus suspension, which was injected in 1 ml. amounts.

Immune Sera.—The rabbit sera used in the neutralization test were prepared by using adjuvants according to the method of Freund (25) as applied to influenza virus by Friedewald (26) except for the exclusion of killed tubercle bacilli. Centrifugal concentrates of the influenza viruses were emulsified in 1 part of Falba and 2 parts of mineral oil (for more detailed information on these substances, see reference 27). A single dose of 4 ml. of the emulsion was injected subcutaneously between the shoulder blades of rabbits weighing between 5 and 6 pounds at the onset of the vaccination. Among 18 rabbits thus immunized none developed abscesses over a period of 8 months. The rabbits were bled at varying intervals and the sera tested for antibodies to the influenza viruses. Maximal titers, as measured by the inhibition of hemagglutination, were reached in about 2 months and the titers stayed constant thereafter for at least 6 months.

EXPERIMENTAL

Upon intra-abdominal injection of certain bacteriologically sterile allantoic fluids infected with influenza virus death was noted within 18 to 96 hours in a varying percentage of the mice depending on the strain of virus, its concentration, and the conditions of cultivation. Thus, 1 ml. of an allantoic fluid containing the F-99 strain was found to cause the death of practically all of the animals, whereas a preparation of the Weiss strain grown under similar conditions and showing equally high titers of active virus in chick embryos was tolerated by all the mice. The following materials served as controls: normal allantoic fluids obtained from embryos of the same age as those used for the production of the infected allantoic fluids; particulate components derived from normal allantoic membranes by differential centrifugation; and, finally, 10 per cent suspensions of infected embryos or chorio-allantoic membranes in saline solution prepared from the same eggs supplying the allantoic fluid. As can be seen in Table I, none of the control materials caused death in mice upon intra-abdominal injection with the exception of the particulate components of normal membranes. In this case a few mice died, but of lesions different from those observed after the administration of influenza viruses. The mice succumbed to the thromboplastic activity of such normal organ particles (28) and long blood clots could be extracted from the inferior vena cava or the right ventricle. The blood of mice dead from the influenza virus injections always was fluid at the time of autopsy. The lethal effect of the virus preparations was observed in several different strains of mice. It was similarly noted upon injection of white rats, guinea pigs, and rabbits.

Results obtained by intravenous injection were similar to those obtained by the intra-abdominal route except that they were more uniform and the lethal

effect was about four times as great. Whereas infected allantoic fluid could be diluted at most two- to fourfold and still produce death in some mice upon intra-abdominal injection, eight- to sixteenfold diluted preparations could cause similar results when injected intravenously. Several experiments supporting this statement are recorded in Table II. In addition, death usually occurred earlier following intravenous injection and mice were found dead on occasion as early as 8 hours after the administration. Since both routes did not seem to differ otherwise in their effect, the data obtained by the various technics will be presented together in the subsequent sections of this paper.

TABLE I

Effect of Intra-Abdominal Injection of Various Specific and Non-Specific Materials

Material injected	ID_{50}/ml. for chick embryos	Results of intra-abdominal injection							
F-99 allantoic fluid	$10^{8.8}$	D_1*	D_1	D_1	D_1	D_1	D_1	D_1	S‡
Weiss allantoic fluid	$10^{9.3}$	S	S	S	S	S	S	S	S
Normal allantoic fluid	0	S	S	S	S	S	S	S	S
F-99 allantoic fluid		D_1	D_1	D_1	D_1	D_2	D_2	D_2	D_2
F-99 10 per cent membrane suspension		S	S	S	S	S	S	S	S
F-99 10 per cent embryo suspension		S	S	S	S	S	S	S	S
Normal membrane particles		D_1§	D_2§	S	S	S	S	S	S

* D_1 = mouse died within 24 hours.

‡ S = mouse survived 10 days.

§ Blood clotted in vena cava.

Pathology

Although differences in the lethal effect of the allantoic fluids containing the various strains of influenza virus were apparent, it could be shown that upon concentration of the agents all strains tested produced death in at least a few of the mice. Thus, there seemed to exist quantitative differences in the effect of individual strains. Furthermore, certain qualitative differences were noted in regard to the pathological lesions produced.

All strains caused severe damage in the liver. This organ was usually of normal size with sharp edges, and the surface showed a fine mottling of varying degree caused by small zones of normally colored liver tissue on a yellowish background of necrotic areas. Histologically, hyperemia and foci of necrosis were found as shown in Fig. 1. These necrotic lesions were found throughout the lobules without preference for either center or periphery. The spleen, likewise, was severly affected in all instances. It usually was enlarged and

dark red in color. On histological examination it showed marked hyperemia and extensive destruction of the Malpighian bodies as shown in Fig. 2.

Besides these lesions common to all strains, others were noted only with some of them. In the case of the F-99, F-12, PR8, and WS strains of influenza A, the blood vessels of the intestinal canal were engorged and the gut was edematous and contained bloody mucous material varying in color from very light to dark red. In some instances clotted blood was noted in the stomach. On the other hand, the Lee and ES strains of influenza B usually did not produce any

TABLE II

Comparison of the Intra-Abdominal and Intravenous Routes for the Injection of Influenza Viruses

Virus (allantoic fluid)			Route of injection											
Strain	Type	Dilution	Intraperitoneal						Intravenous					
PR8	A	Undiluted	D_1*	D_1	D_1	D_2	D_2	d_7‡	D_1	D_1	D_1	D_1	D_1	D_1
		1:2	D_2	d_7	S	S	S	S	D_1	D_1	D_1	D_1	D_1	D_2
		1:4	D_4	d_{10}	S	S	S	S	D_1	D_1	D_1	D_2	D_3	S
		1:8	S	S	S	S	S	S	D_1	d_6	d_7	S	S	S
Lee	B	Undiluted	D_1	D_1	D_2	D_2	D_2	D_2	D_1	D_1	D_1	D_1	D_2	D_2
		1:2	D_2	D_2	D_2	D_3	D_3	S	D_1	D_1	D_2	D_2	D_2	D_2
		1:4	S	S	S	S	S	S	D_2	D_2	D_2	D_2	D_2	D_4
F-99	A	Undiluted	D_1	D_1	D_1	D_1	D_1	D_1						
		1:2	D_2	D_2	D_2	d_6	d_6	d_6	D_1	D_1	D_1	D_1	D_1	D_2
		1:4	D_1	D_4	d_6	d_6	d_8	S	D_1	D_1	D_1	D_1	D_2	d_5
		1:8							D_2	D_2	D_2	D_3	D_4	D_4
		1:16							D_3	D_4	d_5	d_5	d_6	d_6

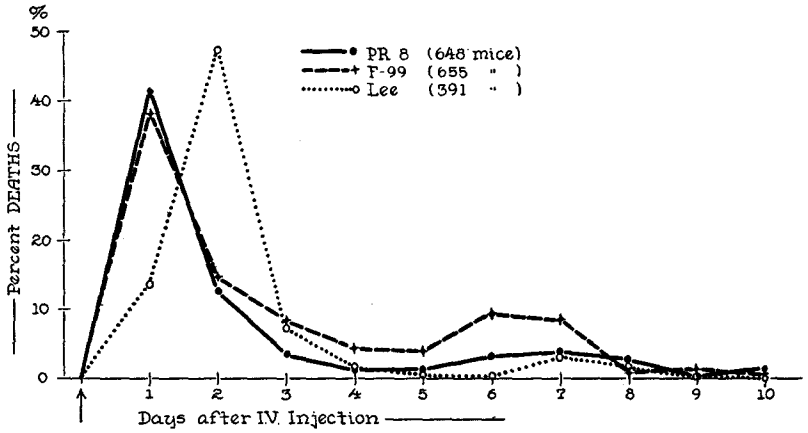
* D_1 = mouse died within 24 hours with extensive damage to organs other than the lungs.
‡ d_7 = mouse died on 7th day with pulmonary lesions.

intestinal reactions but caused the appearance of large amounts of pleural exudate, particularly when the animals survived for 2 or 3 days following the injection. As much as 0.5 ml. of clear yellow fluid has been removed from the pleural cavity of a mouse. This fluid usually coagulated on standing in the test tube. It contained predominantly lymphocytes and only small concentrations of influenza virus as determined by the intranasal inoculation of mice. The lungs of these animals often showed engorgement of the blood vessels.

The difference in the pathological picture between the two groups of viruses was not absolute. The intestinal signs were found on occasion in mice dying from injection of the influenza B strains and pleural exudate was found from

time to time in the influenza A strains mentioned. Other strains of virus (Melbourne and S-15) were found to be intermediate in that either type of lesion occurred simultaneously. The predominance of one or the other type of lesion is also expressed in the curves represented in Text-fig. 1. This figure shows that following the intravenous injection of the PR8 or F-99 strains death occurred mostly within 24 hours, whereas in the case of the Lee strain, the majority of mice died on the 2nd day.

An estimate of the frequency of the occurrence of the major types of acute lesions following the injection of the various strains of influenza virus is recorded in Table III. Other findings in mice which succumbed within the first 4 days after injection of virus included occasional petechial hemorrhages into the wall



TEXT-FIG. 1. Time of death following intravenous injection of three strains of influenza virus.

of the intestinal canal, subperitoneal hemorrhages on the surface of the liver, edema of the pancreas, ascites, and conjunctivitis. In a few instances moribund mice suspended by their tails were found to react with convulsions of the type recorded in the preceding paper, and a few mice were observed in spontaneous convulsion. Other organs showed no particular lesions aside from hyperemia.

In contrast to the acute lesions thus far described, other lesions became apparent in some animals escaping early death. With some strains of virus a new peak of deaths occurred about 6 to 8 days after the injection (Text-fig. 1), which was caused by pulmonary consolidation of the type noted after intranasal infection of mice. This second peak was particularly apparent with the F-99 and F-12 strains, both on intravenous and intra-abdominal administration of the virus, and with the PR8 strain only on intravenous injection (Table III). Such late deaths occurred only rarely with the Lee and ES strains. As will be

shown in a later section of this paper, the virus actually invaded the lung tissue in these cases and multiplied sufficiently to cause these results.

The injection of undiluted infected allantoic fluids usually permitted only a few mice to survive the early lesions. On dilution of the virus preparation, the number of survivors increased and, consequently, more animals were then available to show the development of pulmonary lesions and death therefrom. This is shown in Text-fig. 2 in the case of the PR8 strain. Whereas the percentage of deaths from acute lesions decreased with the dilution of the allantoic fluid preparation injected, the opposite was found in regard to the later deaths caused by the pulmonary infection.

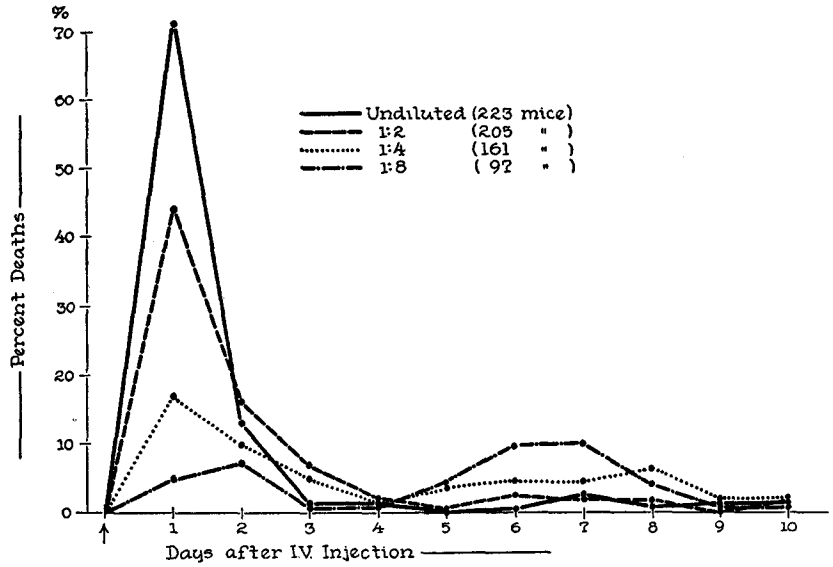
TABLE III

Lesions Following Intraperitoneal or Intravenous Injection of Various Strains of Influenza Virus

Strain	Type	Lesions present in			Pleural exudate	Late pulmonary consolidation
		Liver	Spleen	Intestines		
PR8	A	+++	+++	++	±	++
WS	A	+++	+++	++	±	+
F-99	A	+++	+++	+++	±	+++
Melbourne	A	+++	+++	+	++	++
F-12	A	+++	+++	++	±	++
Lee	B	+++	+++	±	+++	±
ES	B	+++	+++	±	+++	±
S-15	Swine	+++	+++	++	++	±

+++ = lesions regularly present.
++ = lesions frequently present.
+ = lesions occasionally present.
± = lesions rarely present.

In a few instances, usually following injection of the F-12 strain, mice surviving the early lesions developed jaundice 4 to 8 days after intravenous or intra-abdominal injection. The urine of such mice gave positive tests for bilirubin with the Godfried modification of the Harrison spot test (29). The data of one experiment are shown in Table IV. As can be seen, 3 out of the 7 mice escaping early death began to show positive urine tests on the 4th, 5th, and 7th days, respectively. The urine of one of the mice became negative again on the 11th day, that of the second on the 17th day, and the third mouse died when the test was still positive. On autopsy of this mouse, the subcutaneous tissue appeared bright yellow, and the liver of a yellowish brown color. Histologically, the liver showed diffuse and focal proliferation of lymphoid and reticulo-endothelial elements. Multiple foci of necrosis were noted with little or no peripheral reactions.



TEXT-FIG. 2. Time of death following intravenous injection of varying concentrations o influenza A virus (PR8).

TABLE IV

Development of Jaundice in Mice Injected Intra-Abdominally with a Sublethal Dose of Influenza Virus (F-12 Strain)

Virus	Time after inoculation	Harrison spot tests with urine of mouse No.							
		1	2	3	4	5	6	7	8
	days								
F-12 5 times concentrated	2	D							
	3		0	0	0	0	0	0	0
	4		+	0	0	0	0	0	0
	5		+	tr	+	0	0	0	0
	6		0	0	nu*	0	0	nu	0
	7		0	0	+	±	0	0	0
	8		+	0	+	+	0	0	0
	9		+	0	+	+	0	0	D‡
	10		±	0	+	DJ§	0	nu	
	11–20		0	0	+		0	0	
	21		0	0	±		0	0	
	22		0	0	tr		0	0	
					KJ‖				

* nu = no urine obtained.
‡ D = this mouse died from pulmonary lesions.
§ DJ = mouse died; the subcutaneous tissue was bright yellow.
‖ KJ = mouse sacrificed; the subcutaneous tissue was bright yellow.

Attempts to Demonstrate Propagation of the Virus in Liver and Peritoneal Cavity

Since the liver seemed to be the organ most severely affected by the intravenous or intra-abdominal injection of influenza viruses, attempts were made to pass the agents in liver suspensions.

Acutely damaged livers of mice recently dead, or killed when moribund, were emulsified in broth and a 10 per cent suspension was injected intra-abdominally into fresh mice. At the same time, another group of mice was inoculated intranasally under light ether anesthesia to discover, if possible, the presence of influenza virus. Animals injected intra-abdominally were sacrificed after 48 hours and the livers harvested and passed again both by the intra-abdominal and intranasal routes.

TABLE V

Examples of Attempted Intra-Abdominal Passage of Influenza Viruses in Liver Suspension

Passage No.	Strains											
	PR8						F-99					
	Intra-abdominal				Intranasal		Intra-abdominal				Intranasal	
1	D_{18} D_{18} D_{18}						D_{18} D_{18} S_{48}					
	D_{24} D_{24} D_{24}						D_{24} D_{24} D_{24}					
2	K_{48}	K_{48}	K_{48}	K_{48}	3 2 1 ±		K_{48}	K_{48}	K_{48}	K_{48}	d_6 d_6 d_6 d_7	
3	K_{48}	K_{48}	K_{48}	K_{48}	0 0 0 0		K_{48}	K_{48}	K_{48}	K_{48}	0 0 0 0	
4	K_{72}	K_{72}	K_{72}	K_{72}	0 0 0 0		K_{72}	K_{72}	K_{72}	K_{72}	0 0 0 0	

Data of two experiments with the PR8 and the F-99 strains, respectively, are recorded in Table V. All mice survived the intra-abdominal injection of the passage materials for at least 48 to 72 hours. Influenza virus was detectable only in the livers of the first passage, and no longer demonstrable in the subsequent transfers. The F-12, Melbourne, Lee, and S-15 strains have given similar results.

Attempts to pass the agents in livers from animals which developed jaundice at a later date likewise failed. In one such case, a mouse injected intra-abdominally with the F-12 strain developed a positive Harrison spot test in the urine 4 days after injection. The animal was sacrificed on the 5th day and the liver passed in series at 6 day intervals for four passages. A few mice of each passage series were permitted to remain alive. None of them showed any signs and the urine tests were constantly negative.

In studying the concentration of virus in mice at varying intervals after intra-abdominal injection, the data reported by Rickard and Francis could be confirmed (5).

A suitable number of mice were injected intra-abdominally with influenza viruses and four to five mice each sacrificed after ½ to 2, 24, 48 hours, and later. The allantoic fluids were diluted sufficiently to permit most of the mice to survive the early lesions. The peritoneal cavities were washed out with 2 ml. of sterile broth each and the fluids were collected. The livers and lungs were removed and all materials were stored at −15°C. until tests for virus activity were possible. The livers were rinsed with saline solution and then emulsified in broth to form a 10 per cent suspension. The lungs, likewise, were ground and suspended in broth. Titrations of the virus in the various preparations have given the results recorded in Table VI.

The data indicate that the virus concentration is highest in the peritoneal cavity shortly after injection of the allantoic fluid and decreases rapidly there-

TABLE VI

Concentration of Virus in Peritoneal Fluid, Liver, and Lung at Varying Intervals after Intra-Abdominat Injection of Influenza Viruses

Virus			Material	Result of virus titration in chick embryos (ID_{50}/ml.) of materials harvested after				
Strain	Type	ID_{50}/ml.		½–2 hrs.	24 hrs.	48 hrs.	4 days	8 days
PR8	A	$10^{8.7}$	Peritoneal fluid	$10^{8.2}$	$10^{5.0}$	$10^{3.0}$	$<10^{0.5}$	
			Liver	$10^{4.5}$	$10^{1.5}$	$10^{1.8}$	$<10^{1.5}$	
			Lung	$10^{5.0}$	$10^{4.8}$	$10^{5.1}$	$10^{5.1}$	
Lee	B	$10^{8.8}$	Peritoneal fluid	$10^{8.5}$	$10^{4.5}$	$10^{0.5}$	$<10^{0.5}$	
			Liver	$10^{2.8}$	$10^{1.8}$	$<10^{0.5}$	$<10^{0.5}$	
			Lung	$10^{4.5}$	$10^{1.5}$	$10^{3.0}$	$10^{3.0}$	
F-99	A	$10^{7.7}$	Peritoneal fluid	$10^{7.6}$	$10^{4.8}$	$10^{3.3}$	$10^{2.5}$	$<10^{0.5}$
			Liver	$10^{3.3}$	$10^{3.0}$	$10^{2.1}$	$10^{3.7}$	$<10^{1.5}$
			Lung	$10^{3.0}$	$10^{5.7}$	$10^{6.0}$	$10^{7.8}$	n. t.*

* Not tested.

after. No virus could be demonstrated on the 4th day after injection of the PR8 and Lee strains. The F-99 virus disappeared between the 4th and 6th days. The results of the liver titrations were similar. In the case of lung suspensions, the data varied markedly. With the F-99 strain the virus concentration increased, which is consistent with the rather regular appearance of pulmonary lesions and death in mice escaping the early toxic death. In the case of the PR8 and Lee viruses, the concentration of the agents in the lungs varied in the recorded experiments, and in some others. It is felt that this variation may have been caused by the irregularity encountered in the development of pulmonary lesions following the injection of these strains as discussed in a previous section of this paper. In summarizing these experiments it may be stated that no evidence has been found which indicated the propagation of

the influenza viruses either in the peritoneal cavity or in the liver. It is concluded therefore that the lesions encountered are not the result of multiplication of the virus within these tissues but rather the effect of a toxic agent present in the virus preparations.

Relation of the Toxic Agent to the Virus

The relation of the virus to the toxic agent has been studied in a number of different ways, including such divergent technics as the determination of optimal growth conditions, differential centrifugation, and stability tests.

In a previous report (20) it has been shown that the yield of virus and hemagglutinin in the allantoic fluids depended on the concentration of the virus in the seed used for the inoculation of the chick embryos and on the length of the incubation period. The more concentrated inocula produced frequently less active virus and hemagglutinins in the harvest than the more dilute preparations of the same seed. When virus was grown under such varying conditions and the harvested allantoic fluids were tested for toxicity it was noted that the dilute inoculum likewise produced preparations of greater toxicity than the more concentrated seed. A certain correlation between the virus titer and toxicity was therefore indicated. However, the titer of active virus reached its peak frequently within 24 hours of incubation, whereas maximal toxicity was attained usually not before the 2nd day. The infectivity remained high in some instances for 72 hours but the toxicity appeared on occasion to have decreased already at this time. Evidence for these statements is shown in Table VII which summarizes experiments with the PR8 and F-99 strains of influenza A. The differences in the optimal times of harvest imply certain changes in the virus preparation during the incubation period which render it more toxic. On the other hand, toxicity may be on the decline before the infectivity .

Since high dilutions of seed produced the more toxic preparations a study was made to determine whether passage of various strains of virus in high dilution would increase their toxicity. Allantoic passage of the PR8 and Lee viruses in dilution 10^{-7} (approximately 100 ID_{50}) in several passages has increased the regularity with which toxic lesions and death could be obtained, either on intra-abdominal or intravenous administration. However, the last effective dilution of allantoic fluid causing death in about 50 per cent of the mice has not been raised above 1:4 to 1:8 upon intravenous, and 1:2 upon intra-abdominal injection. With the Weiss strain, the toxic activity has not been improved by this measure even on prolonged passage in high dilution and only an occasional mouse was found to succumb to injection of allantoic fluid infected with this strain.

While the foregoing experiments showed that chick embryos with the highest titer of active virus in the allantoic fluid will also develop the highest degree of toxicity, such a correlation does not necessarily imply that the two properties

TABLE VII

Influence of the Concentration of Virus in the Inoculum and the Time of Incubation on the Toxicity of the Allantoic Fluid

Preparation of virus			Properties of the allantoic fluids harvested						
Strain	Dilution of inoculum	Time of incubation	Hemagglutinin titer	Infectivity for chick embryos, ID_{50}/ml.	Route of injection	Undiluted	1:2	1:4	1:8
		hrs.							
F-99	10^{-2}	24	1:128	$10^{9.0}$	I.A.	5/8*			
		48	1:64	$10^{8.2}$		1/8			
		72	1:16	$10^{8.0}$		0/8			
	10^{-4}	24	1:224	$10^{9.7}$	I.A.	2,8			
		48	1:128	$10^{9.0}$		7/8			
		72	1:40	$10^{8.5}$		4/8			
	10^{-6}	24	1:24	$10^{9.0}$	I.A.	0/8			
		48	1:224	$10^{8.9}$		8/8			
		72	1:80	$10^{8.5}$		7/8			
PR8	10^{-1}	12	1:224	$10^{9.2}$	I.V.	4/6	1/6		
		18	1:512	$10^{9.5}$		5/6	1/6		
		24	1:512	$10^{9.2}$		4/6			
		48	1:512	$10^{9.1}$		0/6			
	10^{-3}	18	1:768	$10^{10.3}$	I.V.	6/6	7/10	0/6	
		24	1:512	$10^{9.3}$		6/6	4/6	0/6	
		48	1:768	$10^{9.3}$		6/6	4/6	0/6	
		72	1:512	$10^{9.7}$		6/6	2/6		
	10^{-5}	24	1:768	$10^{10.0}$	I.V.	6/6	5/6	3/6	
		48	1:768	$10^{10.0}$		5/6	6/6	4/6	0/6
		72	1:512	$10^{9.6}$		4/6	5/6	1/6	
	10^{-7}	24	1:128	$10^{10.2}$	I.V.	2/6	1/6		
		48	1:1,024	$10^{10.2}$		6/6	6/6	1/6	0/6
		72	1:1,024	$10^{9.9}$		5/6	4/6	3/6	0/6

I.A. = intra-abdominal.
I.V. = intravenous.
* 5 out of 8 mice died from toxic lesions.

of infectivity and toxicity are part of the same particle. In further experiments employing high speed centrifugation it was found that the toxic agent sediments under the same conditions which also removed the infectivity from suspension. As seen in Table VIII, centrifugation at 20,000 R.P.M. for 20 minutes, which is sufficient to remove more than 90 per cent of the virus as

shown by the hemagglutination test, sedimented enough of the toxic agent to render the supernatant fluids ineffective. The resuspended sediments, on the other hand, contained the toxic agent, although some loss of activity was apparent. Centrifugation of infectious but non-toxic allantoic fluids and resuspension of the sediments in small volumes of saline solution may yield preparations which on intra-abdominal or intravenous injection produce toxic lesions.

Adsorption of the influenza viruses onto chicken red cells and elution therefrom has been used for the concentration of these agents (19, 30). Using this techinc it could be shown that the toxicity of the F-99 strain, for instance, is adsorbed and eluted with the infective agent, as shown in Table IX. After adsorption the supernatant fluid did not cause early toxic death but enough

TABLE VIII

Sedimentation of the Hemagglutinating and Toxic Agents by High Speed Centrifugation

Virus (PR8)			Result of intra-abdominal inoculation of mice
Preparation	Hemagglutinin titer	Dilution	
Allantoic fluid	1:1,280	Undiluted	D_1 D_1 D_1 D_1 D_1 D_1
		1:2	D_1 D_1 D_1 D_1 D_1 D_4
		1:4	D_1 d_5 d_8 S S S
		1:8	d_8 S S S S S
20 min. 20,000 R.P.M. supernate	1:2	Undiluted	S S S S S S
20 min. 20,000 R.P.M. sediment 2 times concentrated	1:3,072	Undiluted	D_1 D_1 D_1 D_1 D_1 D_1
		1:2	D_1 D_1 D_1 D_1 D_1 S
		1:4	D_1 D_1 D_1 S S S
			D_1 D_1 S S S S

virus was left in suspension to initiate propagation of the virus in the lungs and to induce death from pulmonary involvement in the later stage of the experimental period. The eluate, on the other hand, was toxic although some loss of activity was noted.

The experiments indicate that the infectivity and toxicity could not be separated by physical means. Studies on the effect of certain inactivating agents, on the other hand, demonstrated that the infective property is usually more susceptible to the effect of these agents than the toxicity. Table X summarizes a few experiments on the action of heat and of formalin. It can be seen, on heating to 56°C., that the infectivity of one of the Lee preparations decreased from a titer of $10^{-9.7}$ to $10^{-5.7}$, whereas the toxicity end point changed from about 1:4 to undiluted only. Similar results were obtained with formalin 1:2,000 to 1:10,000 if the tests for the activities were conducted in the first few

days after the addition of this agent. More extensive experiments were conducted on the effect of ultraviolet irradiation on these two properties, using a technic previously described (20, 24). As can be seen in Table XI, the infectivity again is more markedly affected than the toxicity although ultimately both properties will be destroyed. In no instance was it possible to obtain preparations which were entirely non-infectious but still toxic to some degree.

Stability of the Toxic Property

The data presented in the previous section were concerned with the resistance of the toxic property to various physical and chemical agents. The next experiments to be described dealt with the stability of the toxic agent under conditions of storage. Table XII summarizes a number of experiments with three strains of influenza virus, determining the stability of the toxic preparations at 4°C. It is apparent that both toxicity and infectivity were remarkably stable under these conditions. The toxicity remained unaltered for 1 to 3 months and decreased slowly thereafter. The infectivity for chick embryos, likewise, remained stable for this length of time. At this time some precipitate had usually formed in the allantoic fluids, which may have been responsible for the loss in activity and which prevented further intravenous tests.

TABLE IX

Adsorption of the Toxic Agent onto and Elution from Chicken Red Cells

Virus (F-99)			Results of intra-abdominal injection of mice
Preparation	Hemagglutinin titer	Dilution	
Allantoic fluid	1:256	Undiluted	D_1 D_1 D_1 D_1 D_2 D_2 D_2 S
		1:2	D_2 d_8 d_9 d_9 S S S S
		1:4	d_6 d_6 S S S S S S
		1:8	d_7 S S S S S S S
Absorbed fluid	1:2	Undiluted	d_5 d_6 d_{11} d_{11} S S S S
Eluate 4 times concentrated	1:1,024	Undiluted	D_1 D_1 D_1 D_1 D_2 S
		1:2	D_2 D_2 D_3 D_3 D_4 S
		1:4	d_6 d_6 S S S S
		1:8	D_4 d_9 S S S S S

The toxic agent withstands repeated freezing and thawing. However, the appearance of precipitates in the allantoic fluids following this procedure usually renders the preparations unsuitable for intravenous injection. Upon dialysis against buffered saline solution prior to freezing the formation of precipitates can be largely avoided. Since dialysis had no effect on the toxic agent, the

preparations can be kept in the frozen state should storage for periods longer than 2 to 3 months be required.

Immunological Data

Tests for the neutralization of the toxic property were performed in chick embryos and mice. The addition of human postvaccination or convalescent

TABLE X

Effects of Heat or Formalin on Infectivity and Toxicity of Influenza Virus Preparations

Strain of virus	Treatment of allantoic fluid	Infectivity for chick embryos, ID_{50}/ml.	Hemagglutinin titer	Intravenous toxicity for mice				
				Undiluted	1:2	1:4	1:8	1:16
PR8	—	$10^{11.0}$	1:256	n. t.	5/6	3/6	0/6	
	5 min. 56°C.	$10^{6.0}$	1:512	0/6				
Lee	—	$10^{9.1}$	1:1,536	6/6	4/6	2/6		
	5 min. 56°C.	$10^{8.3}$	1:1,536	6/6				
	15 " 56"	$10^{6.0}$	1:512	4/6				
Lee	—	$10^{9.7}$	1:192	6/6	6/6	3/6	1/6	
	5 min. 56°C.	$10^{7.4}$	1:192	6/6				
	15 " 56"	$10^{5.7}$	1:128	4/6				
F-99	—	$10^{10.0}$	1:768	n. t.	6/6	5/6	6/6	2/6
	5 min. 56°C.	$10^{8.9}$	1:768	6/6	6/6	3/4		
	15 " 56"	$10^{6.8}$	1:768	2/7				
PR8	—	$10^{10.0}$	1:384	6/6	6/6	3/6		
	Formalin 1:10,000 24 hrs.	$10^{7.9}$	1:384	5/6				
	48 "	$10^{7.8}$	1:256	4/6				
	120 "	$10^{7.0}$	1:512	0/6				
	— 120 " *	$10^{10.0}$	1:512	6/6	5/6	1/6		
F-99	—	$10^{8.8}$	n. t.	6/6	6/6	4/4	4/6	
	Formalin 1:2,000 48 hrs.	$10^{2.0}$	n. t.	1/5				
	1:5,000 48 "	$10^{4.8}$	n. t.	5/5				

* Control repeated after 120 hrs. at 4°C.

sera to influenza virus prevented its multiplication in eggs and the allantoic fluids harvested from these embryos were non-toxic. In the absence of the immune sera highly toxic virus preparations were obtained.

A neutralization test in mice is shown in Table XIII.

To nine parts of allantoic fluid virus, one part of undiluted or diluted rabbit immune serum was added, so that the final dilution of serum amounted to 1:10, 1:100, and so forth. After 30 minutes to 1 hour at room temperature, the mixtures were injected intraperitoneally into six mice each. Intravenous tests require longer incubation of the serum-virus mixture,

possibly on account of dissociation of the two agents on dilution in the early stages of neutralization (31).

As can be seen in the table, high dilutions of anti-PR8 serum prevented death only from the toxic activity of the PR8 strains and, correspondingly, high dilu-

TABLE XI

Effect of Ultraviolet Irradiation on Infectivity and Toxicity

Strain	Time of irradiation	Infectivity for chick embryos, ID_{50}/ml.	Intravenous toxicity for mice			
			Undiluted	1:2	1:4	1:8
	sec.					
PR8	0	$10^{10.7}$	6/6	5/6	1/6	2/6
	60	$10^{7.7}$	6/6			
	180	$10^{4.7}$	1/6			
PR8	0	$10^{10.2}$	6/6	5/6	2/6	1/6
	10	$10^{9.2}$	5/6	5/6	0/6	0/6
	20	$10^{9.0}$	6/6	0/6	1/6	0/6
	30	$10^{7.7}$	3/6	0/6	0/6	
	60	$10^{7.2}$	1/6	1/6	0/6	
	180	$10^{3.5}$	2/6	0/6		
	300	$10^{2.8}$	0/6	0/6		
Lee	0	$10^{9.2}$	6/6	6/6	5/6	1/6
	10	$10^{7.0}$	4/6			
	20	$10^{6.2}$	2/6			
	30	$10^{5.7}$	1/6			
	60	n. t.	0/6			
Lee	0	$10^{9.9}$	4/6	5/6	2/6	1/6
	10	$10^{9.1}$	3/6	6/6	2/6	1/6
	20	$10^{7.0}$	3/6	4/6	1/6	0/6
	30	$10^{5.9}$	4/6	1/6	0/6	
	60	$10^{5.4}$	0/6	0/6		
	180	$<10^{0.7}$	0/6	0/6		
	300	$<10^{0.7}$	0/6			
F-99	0	$10^{9.5}$	6/6	6/6	6/6	1/6
	20	$10^{7.5}$	6/6	3/6		
	60	$10^{5.1}$	5/6			

tions of anti-Lee serum protected only against the toxic effect of the Lee virus. However, some cross-protection was noted in low serum dilutions and similar protective action was noted also with some normal rabbit sera. The difference between the specific and non-specific reaction, however, is sufficiently striking to eliminate any doubt as to the nature of the reaction.

Other experiments showed, furthermore, that strain differences between

TABLE XII

Stability of Infectivity and Intravenous Toxicity at 4°C.

Strain of virus	Test	Dilution	Storage at 4°C.							
			0	2–4 days	1 wk.	2 wks.	1 mo.	2 mos.	3 mos.	4 mos.
PR8	ID_{50}/ml.....		$10^{10.0}$	$10^{10.2}$		$10^{10.1}$	$10^{10.5}$	$10^{9.9}$	$10^{9.1}$	$10^{9.9}$
	Toxicity.....	Undiluted	6/6	6/6		5/5		—	6/6	
		1:2	5/6	5/6		3/5		4/5	6/6	
		1:4	3/6	2/6		—		2/5	4/6	
		1:8	0/6	—		—		—	—	
Lee	ID_{50}/ml.....		$10^{9.3}$		$10^{9.2}$	$10^{9.2}$	$10^{9.0}$	$10^{9.0}$		$10^{8.7}$
	Toxicity.....	Undiluted	6/6		6/6	6/6	6/6	5/6		4/6
		1:2	4/6		5/6	5/6	6/6	4/6		3/6
		1:4	2/6		5/6	1/6	5/6	1/6		1/6
		1:8	—		—	—	1/6	—		—
F-99	ID_{50}/ml.....		$10^{10.2}$	$10^{9.5}$	$10^{9.9}$	$10^{9.0}$	$10^{9.5}$	$10^{9.2}$	$10^{8.1}$	
	Toxicity.....	Undiluted	—	—	—	—	—	—	6/6	
		1:2	6/6	6/6	6/6	6/6	6/6	6/6	2/6	
		1:4	5/6	6/6	6/6	6/6	4/6	4/6	0/6	
		1:8	6/6	1/6	6/6	4/6	4/6	0/6	—	
		1:16	2/6	—	—	—	2/6	0/6	—	
Lee	ID_{50}/ml.....		$10^{9.9}$	$10^{9.4}$	$10^{9.3}$	$10^{9.2}$				
	Toxicity.....	Undiluted	6/6	—	—	6/6				
		1:2	6/6	6/6	6/6	—				
		1:4	3/6	4/6	6/6	—				
		1:8	1/6	1/6	0/6	—				

TABLE XIII

Neutralization of Toxic Activity by Specific Immune Sera

Rabbit immune serum	Final dilution of serum	Strain of test virus											
		PR8						Lee					
		1	2	3	4	5	6	1	2	3	4	5	6
Anti-PR8	1:10							d_{10}	S	S	S	S	S
	1:100	S	S	S	S	S	S	D_3	D_3	D_3	D_3	D_4	S
	1:1,000	S	S	S	S	S	S	D_2	D_2	D_2	D_2	D_4	S
	1:10,000	S	S	S	S	S	S						
	1:100,000	D_1	D_2	D_2	D_2	d_7	S						
Anti-Lee	1:10	d_7	S	S	S	S	S						
	1:100	D_1	D_2	D_2	D_2	d_7	S	d_{10}	S	S	S	S	S
	1:1,000	D_1	D_1	D_1	D_2	D_2	S	d_6	S	S	S	S	S
	1:10,000							D_2	D_2	D_2	D_2	D_2	D_2
Normal	1:10	D_2	D_3	d_6	d_6	S	S	D_2	D_2	D_2	D_2	D_2	D_2
	1:100	D_1	D_2	D_2	D_2	D_3	D_4	D_2	D_2	D_2	D_2	S	S

influenza viruses, apparent in the intranasal neutralization tests, may also be noted in the neutralization of the toxicity. It was found that the F-99 strain was neutralized only to a lesser degree by the anti-PR8 serum, whereas the anti-F-99 serum seemed to be equally effective against both F-99 and PR8 strains. A few neutralization tests with human convalescent and postvaccination sera revealed that antibodies neutralizing the toxic effect of both PR8 and Lee virus may be developed in man as a result of infection or vaccination. Some sera were encountered which in spite of low titers in the inhibition of hemagglutination neutralized the toxic action. This effect, it is felt, may be based on the same protective principle involved in the neutralization by normal rabbit sera. It is possible that this mechanism in turn may be identical with the inhibitory action of low dilutions of sera in the hemagglutination test.

TABLE XIV

Results of Immunization Against the Toxicity of Influenza Viruses When Injected by the Intra-Abdominal and Intravenous Routes
(Summary of Several Experiments)

Vaccine	Test virus	Intra-abdominal route		Intravenous route	
		No. of mice	Per cent deaths	No. of mice	Per cent deaths
PR8	PR8	24	0	84	7
	Lee	24	71	76	95
Lee	PR8	16	50	48	88
	Lee	32	0	49	4
NF*	PR8	20	55	51	92
	Lee	30	66	49	98

* Normal allantoic fluid.

The immunological specificity of the toxic reaction was determined also by the immunization of white mice with influenza vaccines, and subsequent intra-abdominal and intravenous challenge with toxic preparations of the homologous and heterologous strains of virus. Undiluted allantoic fluid vaccines inactivated by ultraviolet irradiation have been used for the immunization of mice in the earlier tests when the intra-abdominal route was employed for the test injection.

The mice were injected twice subcutaneously at a week's interval with 0.1 ml. of vaccine each. One week after the second injection the animals were challenged by the intra-abdominal injection of 1.0 ml. of virus in allantoic fluid.

In these tests toxic activity was not always sufficient to kill all of the control animals immunized with normal allantoic fluid. However, as seen in Table XIV, the pooled results of these experiments show that vaccination protected

only against the homologous toxic agent whereas tests with the heterologous strain gave results similar to those obtained in the controls.

In later tests the intravenous route was employed with essentially similar results (Table XIV). In this case the vaccines could not be injected in undiluted form since anaphylaxis occurred too frequently following the intravenous injection of the test material. However, this untoward reaction could be completely eliminated by the use of vaccines in dilution in broth of 1:10 or greater.

The mice were immunized by two intra-abdominal injections, a week apart, of 0.5 ml. each of the diluted allantoic fluid vaccine and challenged 1 week after the last injection with 1.0 ml. of allantoic fluid given by the intravenous route.

The protection was usually found to be solid when the vaccines were diluted 100- or 1000-fold. A dilution of 1:10,000 frequently protected a good percentage of the mice thus treated. The results obtained were as specific as those reported for the intra-abdominal route of testing.

DISCUSSION

The experiments reported in this and the preceding paper establish the fact that preparations of influenza viruses possess toxic properties. Injected by the intracerebral, intravenous, or intra-abdominal route, they can produce severe lesions in tissues in which the viruses do not usually propagate. The latter fact is supported by unsuccessful attempts of passage of suspensions of the damaged organs and by the failure to demonstrate an increase in virus concentration within the affected tissues. The lesions varied according to the route used for injection but the various activities behaved in a similar manner in respect to all biological, physical, and immunological criteria. The results of the two papers are discussed together, therefore, in this section.

The regularity with which the lethal reactions could be elicited, provided the preparations injected were obtained under appropriate conditions, seems to exclude activation of latent agents. This contention is further strengthened by the absence of such reactions following the injection of various control materials; the demonstration of similar lethal effects on various strains of mice and on other species of animals; the production of somewhat different pathological pictures with the various strains of virus; and the specific neutralization of the phenomenon by high dilutions of immune and convalescent sera; *i. e.*, the neutralization of toxicity of influenza A preparations was accomplished only by anti-influenza A and not by anti-B serum, and conversely.

Most of the above observations exclude also the possibility of accidental contamination of the influenza virus preparations with other agents. This appears unlikely also on account of the fact that all strains of influenza virus, whether mouse- or egg-adapted, have given similar results in at least a few ani-

mals. The ES strain of influenza B gave toxic reactions on second allantoic passage after its isolation from a patient by this route. These considerations showed without doubt that the influenza viruses were responsible for the described reactions. It remained to be analyzed whether the toxic activity was linked to the virus particle or its products, or whether the toxic agent had been formed by the host as a result of infection. The latter possibility could be excluded on account of the type specificity, and even strain specificity, encountered in the neutralization tests. Furthermore, the experiments employing centrifugation and adsorption onto and elution from chicken red cells had shown that the toxicity is intimately linked with the virus particle.

Although it was thus apparent that the virus itself carried the toxic property it could be shown that the toxicity and the ability to multiply are possibly based in part on different constituents within the virus particle. Upon exposure of virus preparations to heat, formalin, or ultraviolet irradiation, the infectivity for chick embryos was lost at a faster rate than the toxic property. Furthermore, the toxicity reached its maximum only after the infectivity attained its peak, indicating certain changes within the culture during the period of incubation.

The observations presented are quite similar to those reported for rickettsial organisms (14, 15), and viruses of the psittacosis–lymphogranuloma venereum group of agents (16). In no instance has it been found possible to separate the toxicity from the infectious agent. It has been suggested, therefore, that these toxic principles should be classified as endotoxins (14, 16). Whether this terminology is correct remains to be seen. Thus far, toxic reactions have been obtained only with the intact infectious agents, and their susceptibility to inactivating procedures, likewise, argue somewhat against this classification.

It is felt that the toxic activity may play an important rôle in the pathogenicity of influenza in man. However, it is not possible at this time to state the exact place of the toxic effect in the clinical picture. The mechanism of infection of mammalian tissues by viruses has not as yet been analyzed. It has been suggested (32) in analogy to observations made with bacterial viruses (Delbrück (33)), that the infective process in influenza involves at least three separate steps: (*a*) the adsorption of the virus onto the susceptible cells; (*b*) entering of the host cell, which leads to certain changes in that cell and prevents other viruses from entering; (*c*) multiplication within the cell. There is no information whatever on the release of the virus from the cells. It is conceivable that the toxicity of the growing virus destroys the cell and thus releases the virus; or, the propagating virus may cause metabolic disturbances within the cell leading to its death; and, finally, the cell may disintegrate as a result of the growing bulk of virus. Toxicity may enter the picture again upon the simultaneous release of large quantities of virus from affected cells. In that case a sufficient amount of virus may enter the blood stream to cause some of the

general signs and symptoms associated with the disease. These various questions should be analyzed in further studies.

The difference in toxicity of various strains of influenza virus may play a rôle in determining the severity of epidemics. Although it is impossible at this time to verify this possibility, a few suggestive observations may be cited. The two strains of virus showing highest toxicity (in terms of minimal effective amount of allantoic fluid) were derived from two fatal cases of influenza, in January, 1939, (F-12) and in December, 1940, (F-99) respectively. The Weiss strain, on the other hand, was isolated by Salk and coworkers (34) from an interepidemic case of influenza A which was particularly mild in character. Since the ES strain has given toxic reactions when only two passages removed from the human source it appears unlikely that the toxic property is acquired in the course of animal passage.

The rôle of the toxic property in questions of immunity to the disease requires study. Experience in experimental infection of human beings (35) suggests that toxic phenomena may enter into the picture of the disease. A study of many pairs of human sera taken before and after infection, or from healthy contacts, and comparison of such data with those obtained by the Hirst technic of inhibition of agglutination of red cells appears worthwhile.

Another application of the findings may lie in the use of the toxicity in the assay of influenza vaccines in mice. The currently used technic employing intranasal testing of the vaccinated animals is hampered by frequent irregularities in the tests, rendering comparative studies of vaccines an extremely difficult problem. It is possible, and preliminary data are in accord with this suggestion, that the intravenous toxicity test may overcome some of these difficulties, provided it can be shown that the two routes of testing measure the same immune response. Such tests are under investigation at present.

SUMMARY

Upon intra-abdominal or intravenous injection of allantoic fluids infected with influenza viruses, mice frequently died within 8 to 96 hours. Similar results were observed upon injection of rabbits, rats, and guinea pigs.

Autopsy of the mice revealed widespread necrosis of liver and spleen, hemorrhages into the intestines, pleural exudation, and other occasional findings. Survivors frequently developed pulmonary consolidation or jaundice. The dominant type of lesion depended on the strain of virus used.

All attempts to demonstrate propagation of the influenza viruses outside of the respiratory tract failed. It was concluded that the early lesions were the result of toxic activities of the virus and not of virus multiplication in the affected tissues.

Injection into chick embryos of highly diluted inocula produced higher titers of virus, hemagglutinin, and toxicity in the allantoic fluids than the use of more

concentrated seed culture. Serial passage of various strains in high dilution frequently increased the toxic activity.

The infectivity often reached its peak in 24 hours when tests for toxicity were still negative. Maximal toxicity was usually not attained before 48 hours.

The toxic activity could not be separated from the infective property by such means as differential centrifugation and adsorption onto and elution from chicken red cells. However, upon heating, formalinization, and irradiation with ultraviolet light, the ability of the agents to propagate was lost at a faster rate than the toxic property.

The toxic property remained stable for 2 to 3 months at 4°C. This stability was comparable to that of the infectivity for chick embryos.

Specific immune sera neutralized in high dilution the toxic activity of the homologous virus. Non-specific neutralization occurred in low dilutions of normal and heterologous immune sera. Strain differences were indicated by this method of testing.

Vaccination of mice by the subcutaneous or intra-abdominal routes protected mice specifically against the toxic effects of intra-abdominally or intravenously injected preparations of virus.

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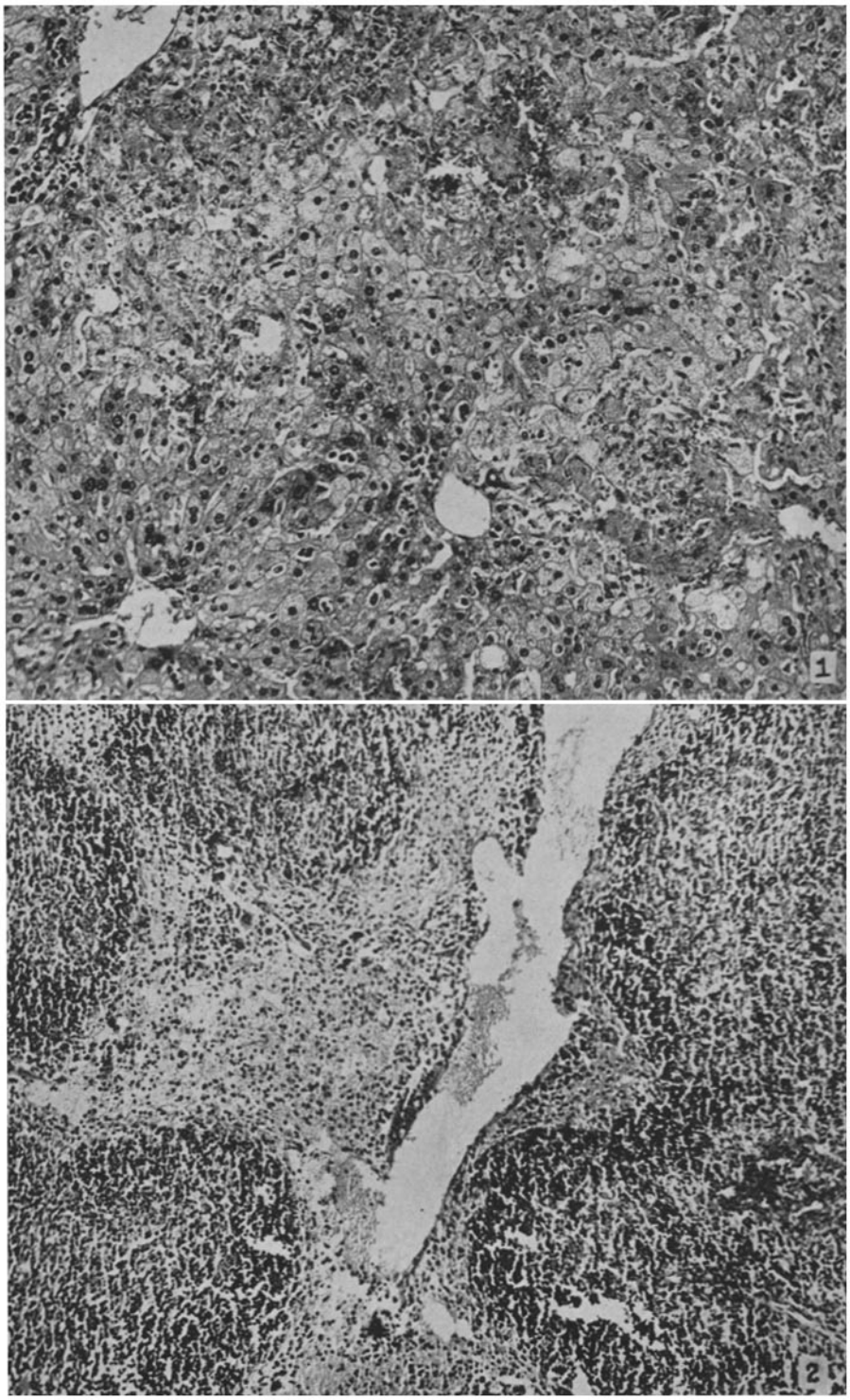
EXPLANATION OF PLATE 25

FIG. 1. Section of the liver of a mouse dying 48 hours after intra-abdominal injection of the F-12 strain of influenza A virus. ×150.

FIG. 2. Section of the spleen of a mouse dying 24 hours after intra-abdominal injection of allantoic fluid infected with the F-99 strain of influenza A virus. ×115.



(Henle and Henle: Toxicity of influenza viruses. II)