SYNERGISTIC ACTION OF HEMOPHILUS INFLUENZAE SUIS AND THE SWINE INFLUENZA VIRUS ON THE CHICK EMBRYO

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PLATES 1 AND 2

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In 1931 Shope (1–3) established that swine influenza was caused by the concerted action of a virus and a bacterium. The clinical and pathological similarities of swine and human influenzas have led several workers (4–6) to suppose that human pandemic influenza might also be caused by two agents acting in concert. But no synergism has been effected in the laboratory animals commonly susceptible to the human influenza virus (7), nor has the inoculation of ferrets and mice simultaneously with *Hemophilus influenzae suis* and swine virus resulted in a more severe disease than that produced by virus alone (8, 9).

Elkeles (10) found pigs to be susceptible to human influenza A virus and showed that the addition of cultures of *H. influenzae*, either human or swine, produced a more severe disease. Shope and Francis (11) corroborated this for the swine *Hemophilus* but found that "the increased severity of the pneumonia produced by the swine virus and bacterium [compared to that of human virus and swine bacterium] seems to constitute a significant difference between the strains of human and swine influenza virus studied." They did not test human strains of *H. influenzae*. Other workers (12) have found that human *H. influenzae* has no enhancing effect on the filtrate disease of swine, whether produced by swine or human virus, but they admit that this may be due to the fact that their strains of human *Hemophilus* fail to persist in the pig.

The lack of evidence for a bacterial component in interpandemic influenza does not preclude the possibility that the pandemics are due to two agents acting in concert, for the two types of disease differ greatly in severity. A bacterial component in pandemic influenza is indicated by the frequency with which *H. influenzae* was obtained in certain Army camps during the epidemic of 1918. Thus the whole question remains open and perhaps will only be settled during the next pandemic.

The work just summed up shows that the failure to obtain a demonstrable synergism for human influenza virus in experimental animals is paralleled by an inability to transpose the complex swine infection to other animals. It seems possible that if the latter disease can be reproduced in a different host this host may prove to be a suitable test animal for tests of synergism in human influenza. The chick embryo is susceptible to infection with a number of species of *Hemophilus*, and the pathological response in all cases mimics the natural disease pattern (13–15). Burnet (16) has demonstrated that the

human influenza virus is pneumotropic in the chick embryo, for it will destroy most of the bronchial alveolar epithelium following intra-amniotic injection.

We have found that the combined infection of embryos with swine influenza virus and *H. influenzae suis* produces a highly lethal infection, while neither one alone kills many embryos. Infection with the virus allows the *Hemophilus* to persist longer than it does in normal embryos. Finally the combined infection has a selective destructive effect on the embryo lungs.

Materials and Methods

All strains of *Hemophilus influenzae suis* and swine influenza virus used in these experiments were kindly furnished by Dr. Shope.

H. influenzae suis strain F was isolated from swine lungworms in 1941. It has been consistently capable of producing swine influenza when combined with the virus. It was used after 30 transfers on blood agar.

H. influenzae suis strain 451 was isolated in 1928 by Lewis and Shope (2). It has been carried on artificial media since then with loss of virulence (17).

The *Hemophilus* was cultured and transferred as routine in 1 cc. of defibrinated horse blood at the base of a plain agar slant.

The swine influenza virus, V 15, was isolated in 1930 and passed through 176 mouse passages.

The PR 8 strain of human influenza virus (influenza A) was isolated from material from Puerto Rico by Francis (18) and is mouse-adapted.

Influenza B (Lee strain) was isolated by Francis (19).

Cultures of *Hemophilus* were killed by heating to 55–60°C. for 30 minutes (20).

Various ages of embryos and methods of inoculation were tested in preliminary trials before 9 day embryos were selected. Some of these tests are shown in Table I. The 9 day embryo has the advantage that the virus infection can persist for a number of days before the embryo gains the ability to regulate its own temperature and before it tends to become naturally resistant to many bacterial and virus infections. Embryos 9 days old were opened by cutting a window in the side of the egg and allowing the exposed chorioallantoic membrane to settle slowly (21). This was inoculated with a drop of the virus suspension, usually either in the form of fresh Berkefeld filtrates of diseased mouse lungs, or allantoic fluid from a previously infected embryo. Occasionally the embryo was inoculated with a saline suspension of an infected chorioallantoic membrane. Control embryos were similarly prepared but not inoculated with virus. The window was covered with Scotch tape and the embryos were incubated for 24 hours, allowing the virus to gain a foothold before the *Hemophilus* was added. One-half of the membranes infected with swine influenza virus were then inoculated with *H. influenzae suis* and reincubated. The control membranes were also inoculated with *Hemophilus*. Each experiment thus contained at least three groups of embryos: those receiving virus alone, those receiving virus and then *Hemophilus*, and those receiving *Hemophilus* alone. When it was desired to test the relative effects of two viruses, five groups were set up, as may be seen in Table II. The presence of *Hemophilus* on the membrane of embryos killed by the combination of bacterium and virus was always demonstrated by film, frequently in culture.

Tests were also made in each experiment for the presence of virus. One method was inoculation of allantoic fluid from one or two of the embryos intranasally into at least two mice under light ether anesthesia. Another test was further embryo passage followed by intranasal mouse inoculation, the test being called positive if typical gross lesions developed in the lungs in 3 to 5 days. The agglutination of the chick's own red blood cells (22) was also useful in detecting the presence of virus, but was only used in conjunction with the above tests.

Later in the study embryos were fixed in Zenker's fixative plus 10 per cent acetic acid and sections stained with hematoxylin and eosin. Only live embryos were used

TABLE I

Lethal Effect of Hemophilus influenzae suis and Swine Influenza Virus within 48 Hours of Inoculation

Mode of inoculation	Age of embryo	Embryo passage of virus	Age of *Hemophilus* culture	Embryos inoculated with: Virus alone		*Hemophilus* alone		Virus + *Hemophilus*	
				No. of embryos		No. of embryos		No. of embryos	
				Dead	Alive	Dead	Alive	Dead	Alive
	days		*hrs.*						
Membrane, simultaneous	10	7	48	0	5	1	4	4	2
Amniotic and allantoic fluid	12	5	72*	0	7	2	4	5	3
Membrane, separate	9	2	48	0	7	0	6	6	1
" "	9	3	48	2	5	2	5	6	0
" "	9	4	24	1	2	2	4	4	0
" "	9	2	24	0	10	1	9	4	6
" "	9	5	36	0	9	0	7	3	5
Totals for 9 day embryos				3	33	5	31	23	12
Mortality, *per cent*				8.4		13.9		65.4	

* 72 hour amniotic fluid from infected embryo by amniotic route.

although in certain cases moribund embryos were fixed to demonstrate the maximum pathological changes. Heart blood cultures were taken from other embryos after immersing them for 1 minute in Zenker's fluid. The chest wall was opened, the heart seared, and punctured with a capillary pipette (23).

RESULTS

Table I shows that the combination of *Hemophilus* and virus consistently kills a greater proportion of embryos than does either one alone. The combined figures also show that the percentage of embryos killed by the combination is three times as great as the sum of those killed by the two agents inoculated separately, so that a synergistic effect is indicated. The mortality figures in Table I and subsequent tables cover a period of 48 hours after inoculation

with *Hemophilus*, but it is to be emphasized that many experiments were observed for several days thereafter and no significant increase in mortality was noticed in any of the series. Actually embryos in the combination series were usually dead within 24 hours after the addition of the *Hemophilus*.

The data in Table I also suggest that the mode of inoculation and the source of *Hemophilus* make little difference. In later experiments the *Hemophilus* cultures were arbitrarily added 1 day after inoculation with the virus.

Certain minor variations in mortality occur from experiment to experiment. Many of these may be due to variations in absorption of toxins from the bacillus, for it was later found that mortality increased if the *Hemophilus* blood suspension was first diluted in saline, so that a greater volume of fluid, containing the same number of organisms, could be added. This diluted suspension covered a larger area of the membrane, presumably allowing for more absorption. A similar effect has been noted in some recent (unpublished) work on the growth of the gonococcus on the chorioallantoic membrane. An increase in mortality was also produced by adding 1 cc. of saline directly onto the drop of the *Hemophilus* blood culture.

TABLE II

Effect of Early and Later Passage of Swine Virus on Mortality of Embryos

Early passage							Late passage				
No. of embryo passage	Virus alone		Virus + *Hemophilus*		*Hemophilus* alone		No. of embryo passage	Virus alone		Virus + *Hemophilus*	
	No. of embryos		No. of embryos		No. of embryos			No. of embryos		No. of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive		Dead	Alive	Dead	Alive
2	0	7	3	5	0	6	9	0	7	5	3
3	1	5	5	1	1	4	11	2	5	6	1
Total.....	1	12	8	6	1	10		2	12	11	4
Mortality, *per cent*.....	7.7		57.1		9.1			14.3		73.3	

The experiments in Table II were carried out to test the possibility that on serial embryo passage the virus of swine influenza lost the ability to act synergistically with *Hemophilus*. The difference in mortality between early and late passages is not statistically significant.

Human Influenza Virus

The synergistic effect in the embryo can only be considered related to the phenomenon in the pig if some degree of specificity is demonstrable. Table III summarizes a series of experiments comparing the effect of inoculating the

same suspension of *H. influenzae suis* on groups of embryos previously infected with human and swine influenza virus. Two representative human strains were studied. Each horizontal line represents a separate experiment. The far greater effect of the swine virus indicates that there is some specificity in the reaction in the embryo, just as there is in the pig.

Recently Isolated and Stock Cultures of Hemophilus

Buddingh and Polk (23) found in work with the meningococcus in the embryo that avirulent stock cultures invaded poorly and killed few embryos, while recently isolated strains invaded tissue and produced septicemia and meningitis. *H. influenzae suis* is apparently rather slow to lose its ability to act in

TABLE III

A Comparison between Human and Swine Influenza Virus

Strain	Human virus					*Hemophilus* alone		Swine virus				
	No. of embryo passage	Virus alone		Virus + *Hemophilus*				No. of embryo passage	Virus alone		Virus + *Hemophilus*	
		No. of embryos		No. of embryos		No. of embryos			No. of embryos		No. of embryos	
		Dead	Alive	Dead	Alive	Dead	Alive		Dead	Alive	Dead	Alive
PR 8	7	0	6	1	5	1	4	3	1	4	5	1
PR 8	2	0	8	4	5	0	8					
PR 8	1	1	7	0	8	2	8	7	2	9	7	4
Lee (B)	1	1	9	1	9	0	10	8	1	6	4	5
" "	3	0	8	1	8	1	8	8	5	4	9	0
Total.....		2	38	7	35	4	38		9	23	25	10
Mortality, *per cent*				16.7							71.4	

concert with the virus in the pig. Strain 451 produced typical swine influenza after more than 175 passages, although it was no longer able to produce influenza by contact (17). Later this strain (No. 451) lost its ability to produce typical influenza when inoculated with the virus (24), and has since been carried on artificial media by Dr. Shope for a total of more than 650 transfers for 14 years. It was compared with the recently isolated swine strain which has consistently produced the complex disease (24). No great difference was found between the two strains when 24 hour cultures were inoculated on identically prepared swine influenza embryos (Table IV).

The establishment of the synergistic action of *Hemophilus* and influenza virus in the embryo is of interest because it allows a study and analysis of some of the factors concerned. From a study of the pathology of swine influenza Shope (6) has suggested the probability "that the activities of both the virus and the organism are influenced by the concomitant presence of the other

agent in the respiratory tract and that both actually contribute to the lesions of swine influenza."

TABLE IV

The Comparative Effect of Recently Isolated and Stock Strains of Hemophilus on Mortality of Embryos

	Recently isolated *Hemophilus*				Virus alone		Old stock *Hemophilus*			
	Alone		+ virus				Alone		+ virus	
	No. of embryos		No. of embryos		No. of embryos		No. of embryos		No. of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
	1	9	4	6	0	10	2	8	2	8
	0	7	3	5	0	9	0	7	3	4
	2	7	8	2	1	9	3	7	8	2
Total.....	3	23	15	13	1	28	5	22	13	14
Mortality, *per cent*.....			53						48	

TABLE V

*Persistence of H. influenzae suis on Normal and Influenza Embryos**

Hemophilus on normal membranes		*Hemophilus* on membranes containing 4th passage swine influenza virus	
48 hrs.		48 hrs.	
Appearance	Film	Appearance	Film
Slightly cloudy	−	Clear	−
" "	−	Ulcer 1.5 cm.	++++
Clear; ulcer 0.5 cm.	−	Slight ulcer	−
Slightly cloudy	−	Ulcer 1.5 cm.	++
Clear	−	" 1 "	++++
Slight ulcer	−	" 2 "	++++
Clear	−	" 3 "	++++
"	−	" 2 "	−
		Dead	+++
		Small ulcer	+++

* 2 drops of an emulsion of *H. influenzae suis* from 10 cc. of saline washings from a 30 hour chocolate agar slant were inoculated on normal and influenza embryos.

Neither virus nor *Hemophilus* was demonstrable by the 5th day after inoculation.

In the experiments outlined in Table I showing the increased mortality produced by the combination of agents, a large number of organisms was almost always used in a small volume of the inoculum (1 or 2 drops of undiluted blood from the standard culture). If fewer organisms are added, or if a dilute suspension of organisms from a chocolate agar slant is added to the membrane

infected with swine influenza virus, few or none of the embryos die. The organisms, however, persist for several days longer than they do on normal embryos and produce larger ulcers with exudate. Table V shows the results of one of the two experiments.

It is evident from these results that infection of the chorioallantoic membrane with the virus of swine influenza predisposes to infection with *H. influenzae suis*.

Effect of Killed Hemophilus

Embryos inoculated with the combination of agents often die 14 to 16 hours after the addition of *Hemophilus*. This suggests that death is due to products of bacterial growth rather than to invasion of the embryos, especially since the

TABLE VI

Effect of Killed Hemophilus on Mortality of Embryos

	Live *Hemophilus*				Virus alone		Dead *Hemophilus*			
	Hemophilus alone		*Hemophilus* + virus				*Hemophilus* alone		*Hemophilus* + virus	
	No. of embryos		No. of embryos		No. of embryos		No. of embryos		No. of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
			4	4	2	5	0	7	3	4
	1	10	5	5	1	10	1	9	9	2
Total.....	1	10	9	9	3	15	1	16	12*	6
Mortality, *per cent*.....			50						66.6	

* Cultures of these embryos were negative for all bacteria including *Hemophilus*.

size of the area covered by the inoculum affects the mortality. Further, blood cultures are usually negative.

To determine the effect of killed bacteria, 24 hour cultures of *H. influenzae suis* were heated at 55–60°C. in a sealed glass tube for ½ hour (20). Embryos infected with swine influenza virus, and normal controls, were inoculated with this emulsion. Control embryos received untreated 24 hour cultures. Results of two groups of experiments are summarized in Table VI. These results show that some product of the bacterial metabolism may act synergistically with the virus.

Pathology

Swine influenza is essentially a lobular pneumonia with a characteristic histopathology. Shope (1) describes it as follows:

"The small bronchi and terminal bronchioles were filled with a polymorphonuclear leucocytic exudate. Bacteria were never numerous in the exudates. . . . There was

an extensive peribronchial round cell infiltration. . . . Alveoli were collapsed and frequently contained desquamated epithelial cells, small numbers of mononuclear wandering cells. Leucocytes and red cells were not found regularly in the alveoli. . . ."

In pigs which have the filtrate disease,

"the bronchial epithelium was damaged, there was a heavy peribronchial cuffing with round cells and the alveolar walls were wrinkled, thickened, and infiltrated by round cells. The collapsed alveoli were usually free of cells and, in contrast to swine influenza, no leucocytes are present, as rule, in the lumen of bronchi or in the alveoli of involved areas of lung" (1).

In the gross the chorioallantoic membranes of embryos infected with swine influenza virus show little unusual other than a slightly edematous thickening and a little whitish exudate on the surface. No definite pocks are noticeable. A few embryos die with extensive hemorrhage and thrombosis.

Histologically the chorioallantoic membranes of 10 to 12 day embryos infected with the virus of swine influenza show several unusual changes. Within the first 2 days a marked and rather extensive phagocytosis of the chick's own red blood cells occurs. Phagocytic cells may be found containing up to 6 or 8 red blood cells each. This occurs usually near a small hemorrhage in the absence of any noticeable inflammatory reaction (Fig. 4). Since phagocytosis is often considered as a foreign body reaction, the obvious explanation is that the red blood cells have been coated with virus, as described by Hirst (22) in the agglutination phenomenon, and are thus foreign to the embryo. However that may be, we have seen this same phagocytosis of red blood cells in 10 day embryos infected with equine encephalomyelitis but have not seen it in other embryo infections.

By the 3rd day of infection with the swine virus the chorioallantoic membrane is considerably altered. Besides the scattered destruction of the ectoderm with a moderate polymorphonuclear response, there are heavy ribbons of infiltrating cells in the mesoderm (Fig. 5). Most of this appears related to blood vessels, either alongside or surrounding them. The predominant cells are mononuclears tightly packed together, with polymorphonuclears dispersed among them. Occasionally the latter occur separately as tight clumps.

We have not noted the foci of ectodermal destruction described by Burnet (25) as pocks. These changes, however, occur in embryo-adapted virus, and we here are dealing with recent embryo passages.

Six days after infection, when virus is no longer demonstrable in the allantoic fluid of the embryo, the membrane may show more chronic changes. The ectoderm is greatly thickened, with layers of cells heaped on top of one another. "Pearls" of ectoderm are swallowed in the chronic inflammatory tissue. Occasionally whole areas of ectocderm with a caseous center are engulfed, with ectodermal cells palisaded around the edge.

Changes in the embryo itself are minor. A few small hemorrhages may occur. The epithelium of the bronchioles in the lung may be a little irregular, but destruction is usually not great and inflammatory reaction is absent (Fig. 1). The occasional moribund embryo shows widespread thrombosis and hemorrhage similar to equine encephalomyelitis in the embryo. Burnet (16) has described severe damage to the embryo lung following intra-amniotic inoculation of human influenza virus, but he also emphasizes the lack of findings after inoculation directly on the chorioallantoic membrane.

Infection with Hemophilus

Gallavan and Goodpasture (13) found that *Hemophilus pertussis* was capable of reproducing the pulmonary lesions of whooping cough when inoculated into the amnion. Later (14) it was found that strains of *H. influenzae* isolated from cases of meningitis frequently caused septicemia and occasionally a meningo-encephalitis in the embryo. The exact relation of these organisms to the *H. influenzae suis* here studied, is difficult to determine.

Two more types of *Hemophilus*, even further removed, have been studied in the embryo. *Hemophilus ducreyi*, the cause of chancroid, induces characteristic lesions in the embryo but cannot readily be carried for more than a few generations. The organisms may be found in large clumps in the infected tissue (15). *Hemophilus gallinarum*, the cause of acute coryza of chickens (26), will produce septicemia in embryos 12 to 13 days old when inoculated into the amnion. Masses of the bacteria may be found both intra- and extracellularly in the infected lung tissue. (Unpublished experiments.)

It is noteworthy that the first three all produce a disease pattern resembling the natural disease. The last, a natural disease of chickens, produces a much more extensive and severe disease in the embryo, for septicemia and pneumonia caused by *H. gallinarum* do not occur in the adult chicken (26).

H. influenzae suis kills only a small proportion of 10 day embryos, and the embryos usually throw off the infection in a few days. Heart blood cultures of thirteen 10 day embryos taken at various intervals have all been negative. Pathological changes in the membrane and embryo are slight. The chorioallantoic membrane is infiltrated with a few polymorphonuclear cells which are usually concentrated in a layer just below the slightly thickened ectoderm. They may be grouped together in nodules. Inflammatory changes are never as marked as in the virus embryos and are practically absent from the central part of the mesoderm. It is difficult to find any bacteria in the sections. A few perivascular foci of polymorphonuclears are occasionally seen in the embryo proper. These have also been noticed in chick embryos infected with human meningeal strains of *H. influenzae* (14). Older (15 to 19 day) embryos are usually not susceptible to *H. influenzae suis*, although 2 of 12 heart blood cultures from 16 day old embryos were positive.

The Combined Infection on the Membrane

Sections of membranes with the complex infection show the same basic pattern as do sections procured after inoculation of the individual components, but the changes are usually more marked. Great masses of mononuclear cells are found around the vessels of the mesoderm. Polymorphonuclears are scattered everywhere. In addition there is frequent thrombosis of the blood vessels, with necrosis of the surrounding tissues. If an ulcer such as those described in Table V is sectioned, the destroyed and necrotic tissue may be seen pushed out onto the surface of the ectoderm. There are masses of *Hemophilus* deep in the base of the ulcer, mostly in the form of short rods. If the embryo survives the infection and recovery sets in, the same general picture described for late infections with the virus alone is seen.

The Combined Infection in the Embryo

The salient histopathological features of the natural disease in the pig are a plugging of small bronchi and bronchioles with polymorphonuclears, a destruction of the bronchial cilia, an extensive peribronchial round cell infiltration, and a collapse of the alveoli with desquamation of the epithelium (1). All of these are reproduced in the chick embryo (Figs. 2 and 3) except destruction of cilia, which are not present until the 14th day. A high mortality has been demonstrated in embryos inoculated on the chorioallantoic membrane with the virus and bacterium of swine influenza. If surviving embryos are studied several days after inoculation, we find a remarkably selective destruction of the embryo lungs. The epithelium of the smaller parabronchia and their adjoining saccules has frequently sloughed off into the lumen, the parabronchia themselves have collapsed and are later virtually obliterated by inflammatory tissue.

The perivascular inflammatory reaction, which is represented in a few scattered foci in embryos receiving *Hemophilus* alone, spreads extensively throughout the lung in the complex infection. Mononuclear cells now predominate near the bronchioles. With the collapse of the parabronchia and bronchioles the whole lung becomes completely overwhelmed by inflammatory tissue so that only a suggestion of the original structure remains (Fig. 3). The skeletons of the parabronchial walls are surrounded and infiltrated by both polymorphonuclear and mononuclear cells. Polymorphonuclears penetrate into the center of the desquamated epithelial mass (Fig. 8). The larger bronchioles are plugged with a polymorphonuclear exudate. Moderate inflammatory changes may even develop in the tubular connections between the embryo lung and the air sacs. The sinuses may also be filled with a similar exudate (Fig. 7) although the destruction of the epithelial lining of the sinuses, like that in ferrets given virus alone (27), is at least not invariably present.

No other organs have shown pathological changes.

It was earlier pointed out that killed cultures of *Hemophilus* could be substituted for the live cultures and would still kill embryos infected with swine influenza virus. Histological examination of an embryo receiving the combination of virus and killed bacterium shows that the killed bacteria will stimulate an outpouring of polymorphonuclears into the parabronchia and larger bronchioles (Fig. 6).

The histological description of these changes is based on examination of sections from 29 embryos, of which 8 received the combination of agents. A more extensive series will be necessary for an accurate description of the pathogenesis of the combined infection. This study, however, establishes the fact that the complex infection is entirely different pathologically from the infections produced by either agent alone.

DISCUSSION

The chick embryo is being used more and more frequently for the study of bacterial and virus infections because, as Goodpasture (28) recently stated, "it seems to have little or no natural immunity of cell types ordinarily susceptible to particular viruses or bacteria in the usual hosts, at least until the last few days of incubation. At certain stages the embryo seems to offer in a way very similar to the natural host specifically favorable environments for the infectious agent."

The present study demonstrates that these statements are also true for a complex infection, caused by a combination of bacterium and virus. The combined infection of the embryo has a mortality several times that of the sum of the individual components. This synergism also has the same specificity that is present in the pig. Finally, the histopathological response mimics the natural disease, for the combined inoculation of the membrane produces a selective destruction of the embryo lungs, thus emphasizing the pneumotropic qualities of the combination.

It is true that Burnet (16) has demonstrated that the virus of human influenza will produce a profound destruction of the embryo lungs *when inoculated into the amnion*, but this type of inoculation admittedly allows the virus immediately to gain access to lung tissue. Inoculation of the chorioallantoic membrane with the swine virus produces lung destruction only if cultures of *Hemophilus* are added.

Only the most tentative and hesitant explanations of this phenomenon can at present be suggested. The swine virus is present in the embryo following chorioallantoic inoculation, even though the changes so produced are minor. The addition of cultures of *Hemophilus* in some way brings out the pathogenic properties of the virus. This may occur by means of some bacterial toxin. The lack of bacteria in the embryo lung proper and the action of the killed bacteria would suggest this. But this cannot be the complete explanation, for

we have demonstrated that infection of embryos with the virus allows the *Hemophilus* to persist longer and to produce larger ulcers.

It would seem that the establishment of the synergistic effect of *H. influenzae suis* and swine virus in the embryo furnishes us with a tool wherewith to study the combined effect of similar agents isolated from human pandemic influenza. With its aid the hypothesis of a complex etiology of human pandemic influenza may be more adequately tested.

SUMMARY

The synergistic effect of *Hemophilus influenzae suis* and swine influenza virus in the pig can be reproduced by the inoculation of these agents on the chorioallantoic membrane of 9 to 10 day old chick embryos. Two strains of human influenza virus that were studied failed to substitute for the swine virus in the synergistic reaction. No loss of synergistic effect was noted when the swine influenza virus was put through 11 chick embryo passages. Recently isolated and old stock strains of *Hemophilus* were equally able to enhance the effect of the virus. Heat-killed cultures of *H. influenzae suis* can be substituted for the bacterial component of the reaction. Infection of the embryo with swine influenza virus predisposes to infection with *H. influenzae suis*.

The combination of *H. influenzae suis* and swine influenza virus causes a selective destruction of the embryo lungs, not produced by the individual components. This pneumonia exhibits the essential features of the natural disease.

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EXPLANATION OF PLATES

The sections were stained with hematoxylin and eosin.

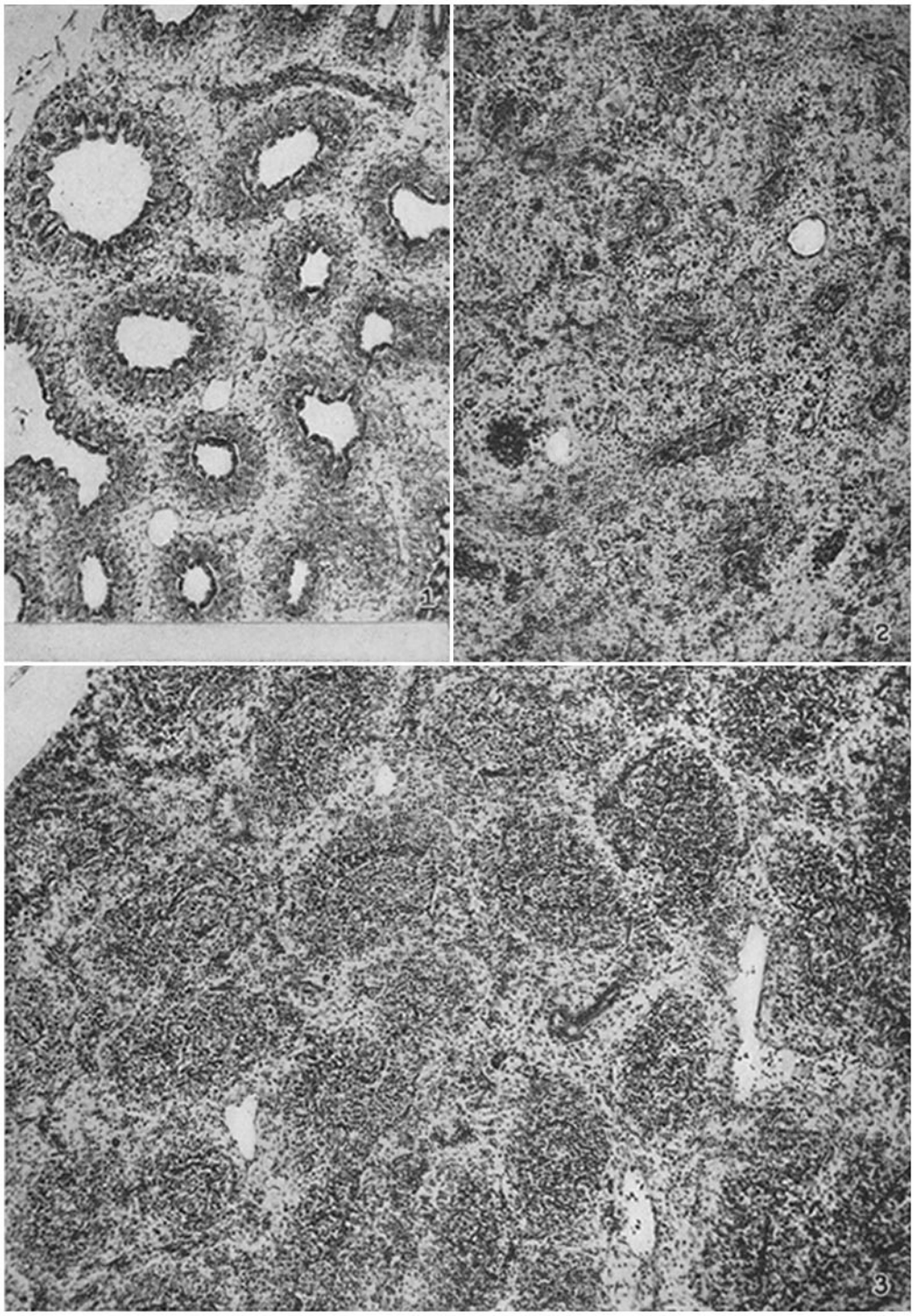
The photographs were made by Mr. J. A. Carlile.

PLATE 1

FIG. 1. Lung from 13 day embryo inoculated on chorioallantoic membrane when 9 days old with swine influenza virus. × 112.

FIG. 2. Lung from 12 day embryo inoculated 3 days previously with swine influenza virus and 36 hours previously with a culture of *Hemophilus*. Both inoculated on membrane. × 112.

FIG. 3. Lung from 13 day embryo inoculated 4 days previously (9 days) with swine influenza virus and 3 days previously with a culture of *H. influenzae suis*. Note complete destruction of bronchi and normal lung structure. × 112.



(Bang: Synergistic action of *Hemophilus* and influenza virus)

PLATE 2

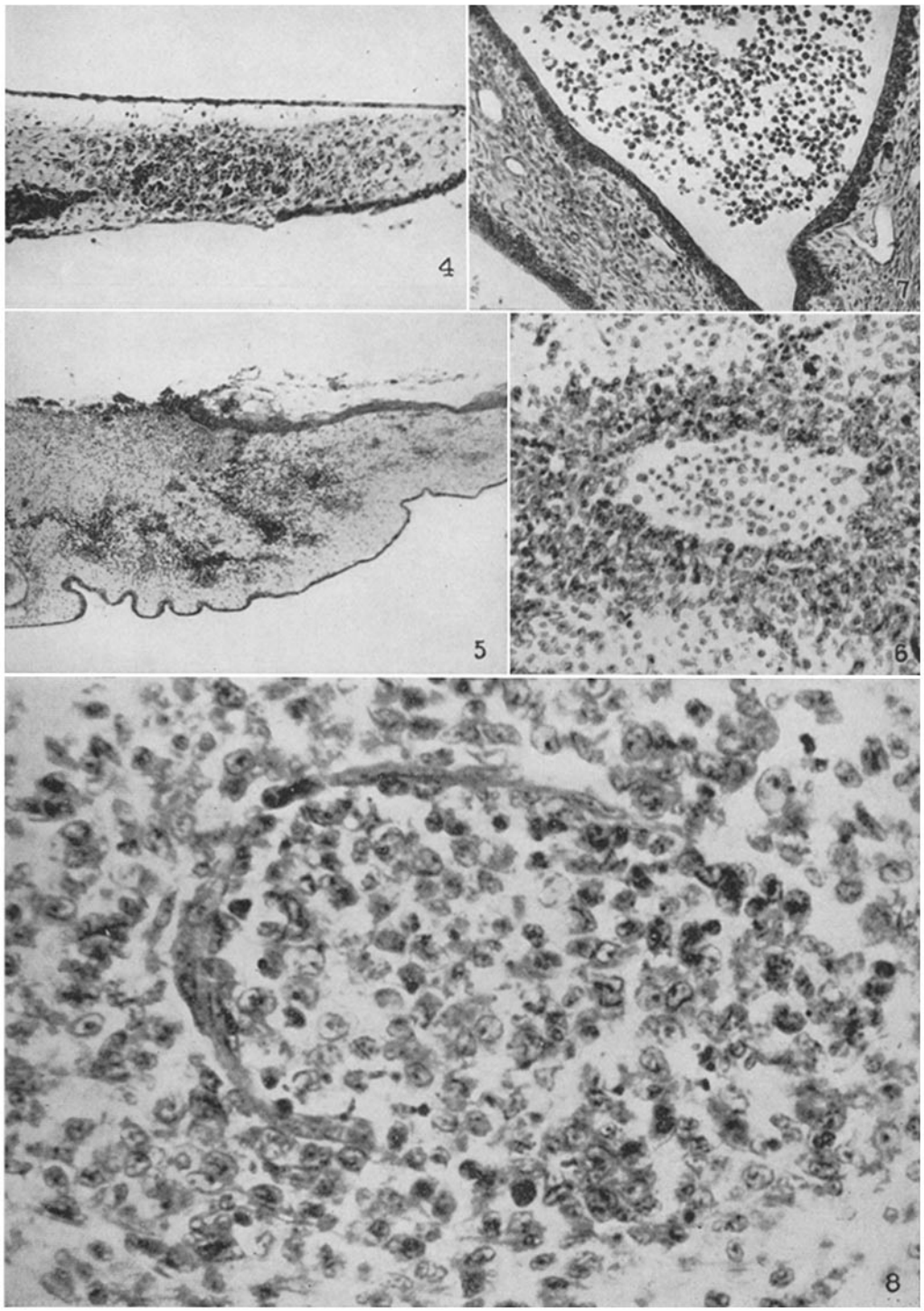
FIG. 4. Chorioallantoic membrane of 12 day embryo infected 2 days previously with swine influenza virus. × 117.

FIG. 5. Chorioallantoic membrane of 13 day embryo infected 3 days previously with swine influenza virus. × 47.

FIG. 6. Lung from 13 day embryo inoculated 4 days previously with swine influenza virus and 3 days previously with killed culture of *Hemophilus*. Note leucocytes in bronchi. × 286.

FIG. 7. Polymorphonuclear exudate in sinuses of 12 day embryo infected 3 days previously with swine influenza virus and 36 hours previously with a culture of *Hemophilus*. Both inoculated on membrane. (Same embryo as in Fig. 2.) × 159.

FIG. 8. Remnants of bronchus in embryo given combination of swine influenza virus and *H. influenzae suis*. Enlargement of Fig. 3. × 804.



(Bang: Synergistic action of *Hemophilus* and influenza virus)