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Some studies on swine influenza: I Comparative study of Hemophilus influenzae suis and Hemophilus influenzae II Antibody response to experimental swine influenza

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SOME STUDIES ON SWINE INFLUENZA

I. COMPARATIVE STUDY OF Hemophilus influenzae suis AND Hemophilus influenzae.

II. ANTIBODY RESPONSE TO EXPERIMENTAL SWINE INFLUENZA.

By

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A Thesis Submitted to the Graduate Faculty for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject - Veterinary Bacteriology

Approved:

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Dean of Graduate College

Iowa State College
1938
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GENERAL INTRODUCTION

Swine influenza is a highly infectious disease of swine of all ages appearing in the fall months of the year and often reaching epizootic proportions. The first epidemiologic reports of the disease were by Koen in Iowa during the fall of 1918, coincidental with the great human pandemic of influenza. The disease has been observed and described at various times since that date, but it was not until 1931 that the etiology of the disease was determined by Shope to be the result of the combined effect of the swine influenza virus and Hemophilus influenzae suis. The synergistic effect of these agents was demonstrated by that investigator when he proved that neither of these agents could reproduce the characteristic disease and pathology when inoculated separately.

The bacterial element of this etiologic complex was studied in 1931 by Lewis and Shope (26) and found to resemble closely the human Hemophilus influenzae organism, originally described by Pfeiffer in 1892. A number of newly isolated strains were available and it was considered desirable to study these organisms in greater detail in order to determine the actual relationship between the swine and human strains.

Numerous reports of the biologic behavior and similarity of the swine virus to the human influenza virus, have been made since the discovery of these two agents. The close relationship and similarity between the two viruses has been established through clinical, epidemiological, and immunologic studies of swine and human influenza and their etiologic agents. In the present study it was considered of value to complement these studies by an analysis of the antibody response of swine to experimental swine influenza and to infections with only the swine influenza virus respectively,
in comparison to literature reports on the antibody response of men to human influenza and to inoculations with the living human influenza virus.
PART I. COMPARATIVE STUDY OF *Hemophilus influenzae suis*

AND *Hemophilus influenzae*.

A. INTRODUCTION

Lewis and Shope (26) studied swine influenza in 1931 and described a hemoglobinophilic organism which they called *Hemophilus influenzae suis* because of its association with the disease and its close biologic, cultural, and serologic similarities to Pfeiffer's *Bacillus influenzae* of man.  

A year later Köbe (18), in Germany, described a similar organism from swine in conjunction with the more chronic disease, "Ferkelgrippe". He called this organism *Bacillus influenzae suis* and considered it also very similar to Pfeiffer's organism. Still later, in 1933, Köbe (19) and Kirchenbauer (16) gave further proof of the organism's similarity to *B. influenzae*. On the other hand, Schlüter (42), in 1936, presented the first evidence showing the existence of a definite serologic difference between human and swine organisms by means of the complement-fixation and the complement-fixation-absorption tests.

Since these were the only reports dealing with the relationships and differences between the swine and human organisms, and since Schlüter's work had been carried out only on the German strain of the swine influenza organism, it was considered of value to restudy Dr. Shope's *Hemophilus influenzae suis* cultures in comparison with *Hemophilus influenzae* cultures. The recent isolation of several new field strains of the swine organism stimulated this interest further, and it brought out, in addition, the possibility of a study of the newly isolated strains as compared to strains isolated as far back as ten years ago. The object was to investigate the cultural and epidemiologic differences in the organisms.
B. EXPERIMENTAL

1. Materials and their Source.

A total of twenty-four *H. influenzae suis* and *H. influenzae* cultures were used through most of the experiment. Fourteen of these cultures were swine strains and ten were human. The *H. influenzae suis* organisms included eight recently isolated strains and six strains isolated during the last decade. Of these, strain 451 was the oldest, having been studied and reported by Lewis and Shope in 1931. The organism had been maintained by 481 weekly culture transfers since its last swine passage. Similarly, three other cultures, strains 791, 1251, and 25 had been maintained for 335, 267, and 97 serial passages respectively. The two youngest cultures in the group were strains 1916 and 24 which had been maintained in the stock culture medium for only 20 and 57 weekly passages respectively.

Most of these strains had been passed through swine at an earlier date, and their culture number originated from the swine passage. These organisms had proven to be pathogenic to swine by being injected in conjunction with the swine influenza virus or by contact infection from an experimentally infected animal. Shope (43), in 1934, reported the loss of pathogenicity of one of the strains as tested by contact infection. Strain 1916, at the beginning of the experiment, proved pathogenic by the method of experimental infection while it had lost its contact infective properties.

The new cultures had been isolated by Shope in Iowa in the fall of 1937 during the swine influenza outbreak and had been maintained through
five weekly passages when used.

The sources of the various swine strains were the following: strains 451, 1916, and 30L were isolated from the lung, strains 24, 1251, 31, 34, 39, and 23 were isolated from the terminal bronchus. The latter, and also cultures 29, 30T, and 35 were isolated from the trachea. Culture 791 was isolated from the brain, while culture 37 was obtained from the nasal exudate of a pig suffering with swine influenza.

The ten human cultures included organisms of different sources and ages, and their general histories for the most part were not available, there being five cultures from Dr. Fothergill, strains 74, 75, 76, 77, and 79, three cultures from Dr. Witebsky, strains 2621, 966, and 3787, and a strain isolated by Dr. Shope, strain "Mayer". Cultures 74 and 79 were supposedly all of respiratory tract origin and all "rough"; culture 3787 was isolated from the sputum of a pneumonia patient; 966 from the chest fluid of a pleuresy patient; 2621 from a meningitis case, and "Mayer" from a throat culture of a "grippe-like" infection.

2. Methods of procedure and Results.

a. Cultural and Morphologic Characteristics.

Literature. The H. influenzae suis organisms have been likened to the H. influenzae organisms because of their cultural and morphologic characteristics by Lewis and Shope (26). These investigators found that their organisms required both of the accessory growth factors described by Davis (6) and Thjötta and Avery (46), and which are said to represent a thermostable iron containing peroxidase, the "X" factor,
and a theromlabile vitamin constituent prevalent in both plant and animal life, the "Y" factor.

The growth of these organisms has been studied both on the chocolate-agar plate and the whole-blood agar plate. In the latter, Lewis and Shope and Kirchenbauer (16) reported on the characteristic satellitic growth of the influenza organisms in symbiosis with non-hemolytic streptococci, staphylococci, and coli organisms. With the chocolate-agar medium, Lewis and Shope found that they considered a difference between the human and swine strains, there being a greater difficulty in adapting swine strains to grow on that medium, as compared to the human strains.

In unheated blood agar both the human and swine strains have been reported as growing poorly. This difficulty has been explained by Krumwiede and Kuttner (22) and Meyer (30) as being due to the inhibitory effect of certain types of whole blood, particularly that of sheep, goats, cows, and to a smaller extent that of man and some horses. Davis (6) and Meyer explained this inhibitory effect as being the function of an oxidizing enzyme present in the blood stream of most of the domestic animals except the pigeon and some horses, and which inactivates the "Y" factor normally present in the blood unless it is destroyed by heat. This difficulty is taken care-of when the organisms are grown symbiotically with any of the previously mentioned organisms.

The morphology of the swine influenza organisms after growth on solid media has been described by Lewis and Shope and Kirchenbauer, as thin bacilli with a certain tendency to thread formation in 24 hours of growth, after which they become increasingly coccoidal. The colonies were relatively small, circular, semi-translucent, and sharply contoured.
The *Hemophilus influenzae* organisms have been classified on a morphologic basis by many authors. Levinthal and Fernbach (25), Kristensen (21), and Klieneberger (17) divided them into typical pathogenic and atypical apathogenic types. The typical types have been observed to form homogeneous smooth colonies consisting of regular cocobacillary forms, while the atypical types have been found to produce heterogeneous colonies of a smaller size, with organisms of various degrees of pleomorphism.

Pittman (32, 33), Holster (12), Kun and Fennyvessy (21), and Platt (32) described the colonies of their "S" type-specific, pathogenic, capsule organisms as opaque, homogeneous, and iridescent, as compared to their "R", "SR", or "O" forms. These changes have also been reported to occur "in vivo" by Hoyle (11) who observed the prevalence of virulent forms, in relation to upper-respiratory infections, while the majority of those isolated from normal throats proved avirulent to mice when injected with "X" and "V" growth accessory factors. Similarly, Dochez, Mills, and Kneeland (7) found that by means of the virus of the common cold in the chimpanzee they could transform "R" forms into "S" forms, which reverted back to the "R" form during periods of health.

**Procedure.** Colony and morphology studies of the swine and human influenza organisms were made on chocolate-agar medium. This was prepared from a basal medium consisting of either a sterilized peptic-digest-beef infusion agar or a beef infusion agar, to which 12 per cent. of filter sterilized defibrinated horse blood was added at a temperature ranging from 80 to 90°C. This was followed by the addition of 6 per cent. sterile potato extract after the medium had cooled slightly, in order to prevent the destruction of the "V" factor. Regarding this
technique, it was observed that when potato extract was added when the medium was too hot, some organisms either did not grow, or grew poorly. This was allied with the differential "Y" factor requirements of individual organisms.

Colony characteristics were studied at daily intervals by means of a binocular microscope provided with an inclinable stage, so that the colonies could be viewed by oblique illumination.

Morphology was studied by smears taken at 24 hour intervals and stained overnight in alkaline Loeffler's methylene blue.

For transfer purposes, the organisms were grown on plain agar slants containing 0.5 to 1 c.c. of sterile defibrinated horse blood. They were transferred once every week.

The cultures tested were isolated three times, in a series, by the single colony method, in order to observe possible differences in cultural behavior and to obtain organisms with more uniform characteristics.

Results. Marked differences were observed in the growth of the different organisms in chocoalte agar. Strains 791 and 1251 were the most difficult to culture, since they did not grow or grew very poorly in some of the lots of media. This difficulty was remedied by the addition of the blood and potato extract to the basal medium at a somewhat lower temperature. These organisms were therefore considered to show higher "Y" factor growth requirements. Still, even with the improved medium those two cultures, as well as strains 23, 24 and 451 showed a delayed growth, as they required over 24 hours to show indications of growth. Strain 1916, on the other hand, showed a good growth in 16 hours.
Of the newly isolated strains, cultures 31 and 34 were the slowest to become adapted to the chocolate agar. After several passages, however, all of the strains presented good growth after the first 24 hours of incubation.

The human strains, as a rule, showed a very rapid and satisfactory growth in the same medium. Strain 2621T, which was isolated from the original 2621 strain, required smaller amounts of the "X" and "Y" growth factors than the 2621H strain for it could be grown in whole-blood agar media and was grown once in a serum medium containing traces of blood.

The colonies of the _H. influenzae_ suis cultures were quite individual, as they ranged from the large flat, "buttery", turbid forms of strain 1916 to the small, raised, hyaline colonies of strain 451. In general, however, most of the swine strains possessed smooth borders and were homogeneously granular. Some of the strains showed individual colonies tending to develop secondary growth on their surface. Upon the isolation of these colonies they soon reverted to the original form. Strains 23 and 37 were the only ones showing pronounced irregularities in some of their colonies, still they could not be separated even though repeated colony isolations were performed. They finally changed so much that they clearly represented "K" types, because of their rough colony characteristics, their constant auto-agglutination and their formation of coarse granules in broth. They were removed from the experiment as it had become impossible to use them in agglutinin-absorption tests.

Of the _H. influenzae_ strains studied, culture 2621 was quite early separated into two distinct and constant types, differing both morpho-
logically and in colony characteristics. Strain 2621H resembled the
swine strains, while strain 2621F resembled more the human strains.
Several of the human strains, culture 3787 in particular, formed colo-
nies with rough borders.

Morphologically, the various organisms did not differ a great deal
from each other. Many of the differences in morphology could be attri-
buted to culture medium differences, as agar-blood slants showed a great-
er prevalence of chains and filaments, while chocolate-agar media pre-
sented a greater percentage of coccosoid and coccosbacillary forms, even
though chains and filaments continued to be present. In agar-blood
slants, it was seen, as already observed by Lewis and Shope, that there
was an increase in the coccosoid forms as the cultures became older.

Culture 1916, even though definitely pathogenic for swine, showed
the greatest pleomorphicity of any of the swine strains, forming long
chains and filaments. Most of the recently isolated strains were also
decidedly pleomorphic.

Of the H. influenzae strains, cultures 966 and 2621F showed the
presence of quite regular short, thick, or fine bipolar rods, while
strain 2621H presented the morphology of many of the swine strains,
namely: medium long, more or less irregular fine rods. Culture 3787
appeared as a highly pleomorphic organism with frequent involution
forms.

b. Biochemic Characteristics.

Literature. Lewis and Shope (26) were the only investiga-
tors to have reported on the carbohydrate fermentation behavior of the
swine influenza organisms. Their findings indicated the lack of
visible fermentation of dextrose, lactose, saccharose, dulcitol, mannitol, glycerol, inulin, and arabinose, when grown in a medium consisting of blood broth, the carbohydrate and brom-cresol purple as the indicator. They found also that their organisms failed to produce indol. Kirchenbauer (16), similarly, failed to find indol formation with the German B. influenzae suis strains.

The literature on the physiologic behavior of the B. influenzae is extensive, in contrast to that of the swine organism. Levinthal (24) and Messerschmidt (29), in 1918 and 1919, reported the fermentation of dextrose and the lack of fermentation of levulose, lactose, mannitol, and saccharose, while maltose and dextrin fermentation was only encountered in some strains.

Stillman and Bourn (45), working on 119 non-hemolytic strains, reported that most of their organisms fermented dextrose, galactose, and levulose. Maltose and saccharose were fermented only by a small percentage of their strains originating from influenza and respiratory tract infections, whereas their normal throat cultures showed fermentation in as many as 60 per cent. of the cases. Their hemolytic B. influenzae strains fermented those two sugars in about 80 per cent. of the cases.

Rivers and Kohn (38), working with 13 meningitis strains and 4 blood strains from pneumonia patients, observed the fermentation of dextrose, galactose, and xylose by most of their strains, while maltose was fermented by only one strain, and saccharose, lactose, mannitol, and some other sugars were not fermented by any of their strains.

Povitsky and Denny (35), in 1921, reported the fermentation of
dextrose by 60 per cent. of their non-hemolytic strains.

Kristensen (21), in 1922, failed to find noticeable fermentation of strains in any carbohydrate, using either liquid or solid media.

Jordan and Reith (15) observed the fermentation of saccharose by many of their non-hemolytic H. influenzae organisms.

Pittman (35) stated that all her "S" strains would ferment dextrose, that galactose was fermented by all her (b) strains and one (a) strain, that xylose was fermented by most (b) strains and not by the (a) strains, and that maltose, saccharose, mannitol, dextrin, levulose, and lactose were not fermented by any of the strains.

Wilkes-Weiss (49), in 1937, found a fermentation of dextrose and xylose by most of her meningeal strains, while her respiratory strains behaved more irregularly, fermenting dextrose in quite a few of the cases, whereas xylose fermentation was observed in fewer strains. Maltose was only attacked by one strain, whereas saccharose, mannitol, inulin, dextrin, rhamnose, and mannose were never fermented by her meningeal or respiratory strains.

Holster (12) found a fermentation of dextrose by both his "S" and "R" strains.

Indol formation has been considered a very important factor since 1920 in the study of H. influenzae. Rivers (36), Stillman and Bourn (45), and Jordan and Reith (15) used this characteristic to a great extent in their classification of the human influenza organisms.

In 1930, Klimeberger (17) found that 60 per cent. of her organisms were indol positive, and she found that there was a predominance of the indol positive forms through the winter months and in children.
Rosen (41), in 1951, found that 71 per cent. of indol positive strains were pathogenic to mice when injected with additional "X" and "V" growth factors, whereas his non-indol forming strains were for the most part non-pathogenic.

In addition, Rivers and Kohn (38) found that 13 of their 14 meningitic strains were indol positive, while Povitsky and Denny (35) found that 75 per cent. of their typical respiratory and meningitis non-hemolytic strains produced indol, while their hemolytic cultures did not produce indol. Rivers and Leuschner (37) reported that 10 of their 15 hemolytic strains did not produce indol. Wilokes-Weiss found that 38 of her 43 meningeal strains were indol positive, whereas only 10 of her 19 respiratory strains produced indol.

Procedure. A total of eight carbohydrates and four alcohols were used in the study of the 24 H. influenzae suis and H. influenzae strains. In addition, three other cultures were tested and not included in Table I. They were: H. influenzae suis strains 1213 and 1491, which were earlier and later respectively, swine passages of strain 1251, and an avian Pasteurella strain, which was used as a simple biological control for the purity of the various sugars in the experiment. The control was satisfactory, as it was found that the organism fermented arabinose, dulcitol, dextrose, saccharose, galactose, sorbitol, and mannitol, without the fermentation of maltose, xylose, lactose, glycerol, lactose, and raffinose, which is according to the usual behavior of the organism, Rosenbusch (40).

The carbohydrates and alcohols used in this experiment were for the most part Pfanstiehl products. The basal medium consisted in a
peptid-digest beef broth, of a pH of 7.2, to which 12 per cent. defibrinated horse blood was added while at a temperature of close to 80°C. The medium was then mixed and filtered through coarse paper. After filtration one per cent. of the sugar and one per cent. of Andrade's indicator were added, and the mixture was sterilised by Seitz filtration.

The carbohydrate tubes were then inoculated from chocolate-agar cultures in order to provide a sufficiently large inoculum. The tubes were then kept at 37°C. and the reaction was read at 24 hour intervals for a space of 10 days. In no case was gas production observed.

Indol production was tested on a similar medium to that used in the carbohydrate tests, but no sugars nor indicator were added. The tubes were then incubated at 37°C. for 4 and 6 days respectively, when the tests were read by first adding 1 to 1.5 c.c. ether, mixing, and by carefully adding Kovacs' (20) indicator along the sides of the tube, to prevent the mixing with the culture. A positive test was characterized by the development of the characteristic purplish-red coloration in the ether zone overlaying the culture.

Results. (See Table I). From the fermentation results on five of the carbohydrates studied it was possible to differentiate the human strains from the swine strains. The similarity in behavior of the swine strains was quite apparent from the study of those carbohydrates. The human strains in general behaved somewhat more irregularly, and this was especially the case with the "Mayer" strain, which proved quite distinct by its degree as well as type of fermentation.

Xylose fermentation did not occur in any of the swine strains,
while it did in most of the human strains, in which the reaction occurred within the first 36 hours and was quite marked.

Dextrose fermentation occurred in 8 out of 10 of the human strains, while it occurred in only 2 of the swine strains, there being only traces of the reaction in strain 451. The reaction occurred within the first 36 hours and was quite marked in most of the cases.

The disaccharides maltose and saccharose, on the other hand, were fermented by all the swine strains and by only one of the human strains. The reaction, which was quite pronounced with some strains, occurred in maltose in 3 to 5 days, while in saccharose the reaction was for the most part very small and appeared on the 2nd to 5th days of inoculation.

Galactose was fermented by 9 out of 10 of the human strains in 2 to 3 days, while only one of the swine strains showed a fermentation of this sugar.

Neither lactose, arabinose, raffinose, glycerol, nor dulsitol were fermented by either of the strains, while mannitol and sorbitol showed traces of fermentation in 4 and 2 swine strains respectively.

Indol was only formed at both the 4th and 6th days by the human strain, and then only in 5 of the 10 strains studied.
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**TABLE I**

Biochemical characteristics of **Streptococcus faecalis**
c. Serologic Characteristics.

Literature. Lewis and Shope (26) and Kirchhambauer (16) reported, after cross-agglutination and agglutinin-absorption studies, that swine influenza strains were of a heterogeneous serologic nature. Most of their strains showed mainly a homologous type of agglutination. The former authors obtained their results from rabbit sera with agglutination titres ranging from 1:400 to 1:800, while the latter obtained theirs from sera with titres of not over 1:600. Kirchhambauer obtained his sera by inoculating his rabbits five times with living organisms at four day intervals. He prepared only sera for the swine strains and his normal serum showed titres as high as 1:50, which was for the most part the extent of the agglutination titres of the human strains with the swine-strain immune sera.

Lewis and Shope showed that some of their H. influenzae suis sera showed agglutination titres of 1:200 with some of the human strains, and that inversely a H. influenzae serum of a high homologous titre showed agglutinins towards most of the H. influenzae suis strains, in titres ranging from 1:200 to 1:600.

Schlüter (42), in 1936, on the other hand, found that he could differentiate distinctly the swine strains from the human strains by his studies with the complement-fixation and complement-fixation-absorption tests on these organisms. He used, for that purpose, three German B. influenzae suis and three B. influenzae rabbit immune sera, prepared by the application of 7 injections of heat killed organisms at intervals. He succeeded also in differentiating these organisms from B. pertussis and B. influenzae anseris by the same tests.
The *H. influenzae* serology literature is very voluminous and is crowded with controversial reports dealing with the type of antigenic nature of this organism.

Roos (39), Huntoon and Hannum (13), Gay and Harris (10), and Small and Dickson (44) reported that the organisms were very closely related and that their differences were only of degree. Their studies were based on a few immune sera of low agglutination titres. However, there are numerous other reports stating the *H. influenzae* organisms to be extremely heterogeneous in their serologic behavior.

Wollstein (50), in 1915, and Valentine and Cooper (48), in 1919, were the first to report the extreme serologic individuality of these organisms. The latter authors emphasised this fact with their finding six different serologic types of *H. influenzae* in one family during the 1918 influenza epidemic. Because of that finding they questioned the etiologic relationship of the organism to epidemic influenza.

Other authors who reported similar findings were Bell (2), Bieling (3), Coca and Kelley (5), Maitland and Cameron (28), Utheim (47), Kristensen (21), Yabe (61), Povitsky and Denny (35), Jordan and Sharp (14), and Klieneberger (17), all of whom reported on the great heterogeneity of the organism. Some of these authors isolated as many as 5 different serologic strains from the same patient.

Other evidences of the serologic irregularity of the *H. influenzae* organisms were provided by Povitsky and Denny with their findings on the agglutination differences between individual colonies of the organism. Similar findings were reported by Lubinski (27), Pennyvesey and
Kopp (8), and by Pfeiffer (31). They encountered differences in agglutination degree and titre, as well as differences in the occurrence of zones, from different colonies, cultures from different parts of the body, and from different cultural or animal passages. These authors concluded that the "receptor apparatus" of H. influenzae was open to rapid and marked changes, which has been reported also, even though to a lesser extent, in such organisms as B. paratyphosus B and Vibrio cholerae.

Chesney (4) and Fovitsky and Denny (35) found that only one-third of their organisms were related serologically, while the remaining were of a heterogeneous nature.

Yagi (52), in 1955, found that by the agglutination and agglutinin-absorption tests 10 of his 50 strains would fall into two groups, while the remaining would react completely heterogeneously.

Wilakes-Weiss (48a), in 1937, found by cross-agglutination and agglutinin-absorption tests that 14 respiratory H. influenzae strains were highly heterogeneous.

Bailey and Shorb (1), in 1930, were able to subdivide 61 strains into three main groups, of which the second included most of the hemolytic strains, by means of the complement-fixation test.

Pittman (33) found by agglutination and precipitation tests that 15 strains, which she called "type-specific" or "S" strains, consisted of two serologically distinct groups, which she later increased to six (see Platt (32)), while the "R" strains agglutinated with all the "R" sera, having lost entirely their type specificity.

Fothergill and Chandler (9), who worked on 150 meningeal strains,
confirmed Pittman's findings regarding the type specificity and homogeneity of the "S" strains. The "R" strains obtained by growing on repeated transfers in anti-"S" serum media behaved serologically in a non-specific and irregular manner. None of the "S" strains agglutinated with the "R" strain sera, which had also been observed by Pittman.

Platt (34), stated that his "S" nasopharyngeal strains showed such an irregular relationship by agglutination and agglutinin-absorption tests that he was unable to identify them from "R" strains. He was able to subdivide his "S" strains only after the partial purification of their soluble substances, followed by the use of precipitation tests. Contrary to Pittman he found a grouping tendency in his "R" strains when tested against "S" sera.

Of all the H. influenzae strains mentioned in the literature, the meningial strains have been shown to be the most serologically homogeneous. This has been shown by Povitsky and Denny, by Rivers and Kohn (38), by Pittman, whose seven meningial strains belonged to one group, by Pothergill and Chandler, who reported that 95 per cent. of their 150 meningial strains were of the same serologic type and by Wilokes-Weiss (48a) who observed the presence of five main cross-agglutination and agglutinin-absorption groups out of twenty-eight meningial strains.

Procedure.

(1) Agglutination Technic. All the previous serologic work on the swine influenza organism has been done with rabbit immune sera of relatively low titres. It was considered wise, therefore, to prolong the immunization of the 16 rabbits in these experiments until the homologous agglutination titres reached an average value of 1:2500.
Rabbit Antigens. Six swine and two human strains were used as antigens. The antigens were prepared from cotton-filtered saline washings of 24 to 48 hour chocolate-agar slants in large potato tubes. The antigens were removed and then washed three times in physiologic salt solution with intermediate centrifugations at 1800 revolutions per minute, and the antigen made up to a one per cent. concentration, by volume. Since it had been determined that the organisms remained viable in physiologic salt solution for 48 hours, the same antigens were used for each series of three injections. The antigens were kept in an ice box between inoculations.

Immunization of Rabbits. Sixteen rabbits were immunized, two rabbits for each antigen, with 1 c.c. doses of the antigen. Three daily injections were given, followed by an interval of 4 to 6 days after which three more daily injections were made. The alternate periods of injections and rest were followed until a total of 27 injections were given to the animals through a period of 50 days. They were then bled from the ear vein or from the heart, the serum separated and sterilized by Seitz filtration. The normal cross-agglutination titres of the sera before the beginning of the experiment ranged between 0 and 1:400 in all sera. Cultures 1916, 24, 25, and 2621T showed the highest titres with most of the normal sera. The titre of the sera at the 16th injection ranged between 1:640 and 1:4720.

Agglutination Antigens. These antigens were prepared by growing the organisms in the same way as the rabbit antigens. They were then washed with salt solutions of different concentrations to which was added 0.5 per cent. phenol. The salt concentration used depended
on the "salt optimum" of each individual strain, as tested in a series of preliminary experiments. Cultures 1916, 24, and 29 required a 0.5 per cent. salt concentration and cultures 23 and 2621f required a buffered 0.2 per cent. salt concentration, while the remaining cultures were prepared in the 0.85 per cent. salt concentration. The optimum salt concentration for some of the cultures varied through the three months of the experiment, so that different salt concentrations were used during the different periods of the experiment. The purpose of these changes in many instances was to control auto-agglutination, which was a constant problem with some of the strains. This was especially the case with strains 25 and 37, which, after some time, became so persistently auto-agglutinating that they were eliminated.

The antigens were prepared to contain 0.1 per cent. dead organisms by volume.

Agglutination Tests. The agglutination technic for *H. influenzae* has been studied by many investigators, as it has always been a source of great difficulty, either because of auto-agglutination, complete lack of agglutination, or due to peculiarities in behavior of the organism at different temperatures. Rivers and Kohn (38) studied the organisms by incubating the agglutination tubes at 55°C. for 6 hours. Lewis and Shope (26) and Krohwobauer (16) carried their agglutinations for 2 hours at 55° and 56°C. respectively, which was thought to prevent auto-agglutination. Pittman (33) and Platt (34) working with "S" and "R" strains found that type specificity was greatly influenced by the temperature and period of incubation. They preferably used incubations of one to two hours in a water bath at 37°C.
Incubation at 55°C. destroyed, to a considerable extent, the type
specificity of their organisms, causing most of them to show cross-
agglutination at high titres.

In this experiment the tests were incubated in a 55°C. water bath
for 4 hours, after which the tubes were placed in an ice box over night.
The different sera were tested in dilutions from 1:20 to 1:20,480. The
agglutination tests were then read with the assistance of a strong
light, as the agglutination of this organism was difficult to differen-
tiate from the frequent normal bacterial sedimentation, as in many strains
the agglutination granules were very small. Numerous controls were
used to facilitate the recognition of auto-agglutination.

(2) Absorption Technique. Each of the immune sera was diluted
with physiologic salt solution to a concentration of 1:20 to make a
total of 10 c.c. The sera were then placed in centrifuge tubes and
1.25 c.c. of a 10 per cent. suspension of the washed homologous organ-
isms was added to each tube. The tubes were then mixed and kept in a
water bath at 55°C. for 4 hours, followed by refrigeration over night
and then centrifuged at 1800 revolutions per minute until the superma-
tant fluid was clear. The individual sera were then tested for homolo-
gous agglutination. Those which still showed homologous antibodies
received an additional amount of the 10 per cent. bacterial suspension
and the same absorption process was repeated.

The so-absorbed sera were then studied by cross-agglutination
tests with all the organisms. The dilutions began with a 1:2 dilu-
tion of these sera. Since the sera had already been diluted 1:20 the
first dilution tested was of 1:40.
Results. The control agglutination titres were quite high, ranging from 1:20 to 1:400, so it was thought wise to obtain immune titres as high as possible, in order to allow a safe margin for reading antibody differences.

The final titres of the cross-agglutination tests (see Table II), ranged from 1:320 to 1:20,480 for the tests of the swine sera and swine strains.

From the homologous agglutination results of the various sera, it was seen that the various organisms used in the immunization of the rabbits differed markedly in antigenicity. Sera 1916 and 24 presented the highest titres. These were followed by sera 1251 and 791, and then by 451 and 23.

By cross-agglutination it was seen that the human sera showed relatively low titres against the swine strains, while their homologous titres were high.

There were indications also of a quite distinct behavior of the swine strains as compared to the human strains. The former, with a greater homogeneity, even though there were distinct signs of individuality between the various strains. At the same time, it was seen that the human strains were markedly heterogeneous and individual in their cross-agglutination behavior.

Comparing the agglutination titres of the newer strains to the older, strains 29, 31, and 34 presented the lowest titres, while strains 30L, 30T, 35, 57, and 59 presented the highest titres in the group. It was interesting to note in that connection, that in three of the swine sera there were cross-agglutination titres exceeding the value of their homologous strains.
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**Legend:** Number indicates extinction time following a given exposure.

**Table IX**

Cross-extinction results.
Of the *H. influenzae* strains, only one, strain 76, behaved similarly to the swine strains, the remainder showed either no agglutination titres or their titres were very low.

The homologous agglutinin-absorption tests (see Tables III and IV), emphasized still further the difference between *H. influenzae suis* and *H. influenzae*. For the most part, swine strains removed almost all of the antibodies of the swine sera against the swine strains, while they removed little or none of the antibodies for the human strains. On the other hand, the homologously absorbed human sera absorbed only relatively small amounts of swine antibodies, while their power of absorption of human antibodies was increased.

The serologic individuality of the various organisms was noticed by the agglutinin-absorption tests even more than in the cross-agglutination tests, and here again the human strains proved much more heterogeneous.

Strain 76, which resembled a swine strain in the cross-agglutination tests, appeared entirely different by the agglutinin-absorption test. Colony isolations 2621H and 2621T, which had presented the same homologous titre, proved to be distinct by absorption of the original 2621 serum with each of the colony isolations respectively.

It was also of interest to note that in the swine group there was a tendency toward subgrouping (see Table IV), as the first six strains showed fairly high agglutination titres against the homologously absorbed 451, 1251, and 791 sera, while the last six strains showed almost no agglutination antibodies remaining in any of the swine sera, while the titres remained the same with the human sera.

The original normal rabbit cross-agglutination titres were compared
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</tbody>
</table>

**Legend:**
- no absorption
- incomplete absorption
- small absorption (75-85%) if present of
- (90-85%)

**Homologous Absorbed Serum**

Degree of homologous absorption of sera and human influenza strains.
TABLE IV

Cross-agglutination of Homologous Absorbed Sera.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Swine Sera</th>
<th>Human Sera</th>
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<tbody>
<tr>
<td></td>
<td>24 1916</td>
<td>451 1251 791</td>
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<tr>
<td>Swine</td>
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<tr>
<td>24</td>
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<tr>
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<td>966</td>
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<tr>
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<td>2621H 320</td>
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<td>Mayer</td>
<td>- -</td>
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Legend: Numbers indicate titre endpoints. 
(*) = no original agglutination.
with the figures given in Tables III and IV and they did not affect
to any significant extent the degree of absorption nor the extent of
cross-agglutination of the homologously absorbed sera.

Precipitation tests were performed with the supernatant fluids
of most of the cultures tested, but no precipitating antibodies were
observed. Pittman's (33) precipitation test procedure was employed
in this part of the experiment. The failure to observe precipitins
may have been due to the technic used in preparing the rabbit antigens,
which were washed three times before being injected into the animals.
C. SUMMARY AND DISCUSSION

The comparison of the few *H. influenzae suis* and *H. influenzae* organisms studied in this experiment revealed that both showed distinct differences in carbohydrate fermentation, and to a somewhat small degree, in serologic behavior. There were also, in many instances, differences in indol production. In comparing these differences with the literature dealing with the relationship of the respiratory and meningeal strains of *H. influenzae*, it was seen, even though the present report is on the basis of a small sample, especially of human strains, which have always proven very heterogeneous, that the difference between the swine and human strains has proven greater than that reported in the literature between the meningeal and respiratory strains. Whether these differences are marked enough to warrant a separate species classification for the swine organisms is still a question, at least until additional data have compared the swine organism with more human strains, and especially with influenza pandemic strains. However, the study of the literature describing the carbohydrate fermentation behavior of the *H. influenzae* strains isolated during the 1918 influenza epidemic, with the less perfected isolation methods used at the time, as reported by Stillman and Bourn (45), Messerschmidt, Hundeshagen, and Scheer (29), and Jordan and Reith (15), demonstrated that even during the epidemic years *H. influenzae* differed from the present fermentation results for *H. influenzae suis*. The above investigators encountered the fermentation of saccharose and maltose by 25 to 30 per cent. of their strains, while it occurred in all of the swine strains studied in this experiment. Still, this was accompanied by the fermentation of dextrose and galactose by a majority of their strains, which is not the case with any of the swine
strains of the present study.

The fact that indol was produced by 50 to 70 per cent. of the human strains while the swine strains have never been shown nor have been reported to have shown indol production either in the United States or Germany supplies additional, even though only partial evidence, that the two organisms differ from each other.

Human and swine organisms have been reported as similar by Lewis and Shope (26) and Kirchenbauer (16) who worked with the swine organism of the more chronic disease "Ferkelgrippe", on the basis of irregular antigenic behavior as well as serologic relationships. However, Schlüter (42), was able to differentiate the German swine influenza organism from H. influenzae by means of the complement-fixation and complement-fixation-absorption tests.

In these experiments a somewhat similar difference was observed between the swine and human strains, on the basis of agglutination and agglutinin absorption tests with immune sera of high antibody titres. The agglutination differences in some cases were only quantitative, but they were usually of sufficient significance to establish a noticeable distinction between the swine group as a whole and the individual human strains. Wherever the unabsorbed cross-agglutination results were insufficient in demonstrating this difference, the homologously absorbed cross-agglutination reactions provided further evidence differentiating the swine from the human organisms.

Agglutination and agglutinin absorption tests of the human and swine strains showed the individuality in behavior reported by other investigators, but in these experiments those factors of heterogeneity were much less pronounced in the H. influenzae suis strains. The swine organisms, even though showing a partial individuality in behavior, appeared to be com-
posed of strains acting as two closely related subgroups based on the cross-agglutination results of their homologously absorbed sera. However, this subdivision must be considered preliminary, since the different strains have not been tested by heterologous absorption experiments.

This grouping tendency of the swine strains could not be correlated definitely with other characteristics. Three of the members of this group, strains 451, 1251, and 791 were old cultures, and their "V" growth factor requirements were the greatest of the whole group. On the other hand, three other cultures, SOL, 30L, and 35, had been isolated recently from field swine influenza cases.

No constant morphologic nor cultural differences have been observed between the swine and human organisms, other than possibly a greater morphologic similarity between the swine strains, as compared to the human strains.

Of the cultures tested, two of the swine strains behaved as possible "R" forms, on the basis of Holster's (12) description of the "R" and "S" forms. These two cultures presented evidences of "rough" colony characteristics. In addition they flocculated in liquid media and were constant auto-agglutinators notwithstanding the use of different salt concentrations and buffer solutions. One of the cultures had been recently isolated from the nasal exudate of a pig with swine influenza, while the other, strain 23, was an older strain, which had been passed through 97 weekly transfers. The latter was included in subgroup 2 on the basis of the serologic behavior of its homologously absorbed immune serum.

In the interpretation of the agglutination and agglutinin absorption tests, the normal rabbit agglutination titres, length of immunization, and the effect of high agglutination temperatures had to be taken into consid-
eration, since they possibly contributed to the loss or decrease in type specificity of the organisms studied, as previously reported by Pittman (33) and Platt (34). In this experiment, however, these specific secondary antigenic influences demonstrate, to a possible maximum extent, the degree of antigenic relationship between all the organisms studied. On the other hand the cross-agglutination studies on the homologously absorbed sera prepared at the same temperature and length of incubation as the agglutination tests, tended to eliminate all those primary antibodies which had established the close cross-agglutination relations between the organisms. This procedure left only those antibodies which were possibly the real secondary antibodies denoting group or type differences.

Since Pittman's type-specificity is evidently based on a soluble antigenic fraction of her "S" organisms, which was not encountered in the swine organisms of the present study, it is possible to consider that the basis of the serologic relationship or individuality of, at least, the H. influ-

enzae suis organisms, resides on one or various somatic antigens or "antigenic fractions". Some of these antigens or "antigenic fractions" seem to produce antibodies which become evident at a slower rate, and for which the homologous antigens are present in very small amounts during absorption process, so that they remain unabsorbed, even though a supposedly complete homologous absorption has been performed. The complexity of that antigen or "antigenic fraction" is easily demonstrated by the extent of individuality of both the unabsorbed and homologously absorbed cross-agglutination titres, as determined by the type and degree of agglutination of the same serum towards different organisms.
During the course of this study it has become apparent that a
future study on the pathogenicity of the H. influenzae suis organisms
following the technics devised by either Rosher (41) or Hoyle (11), or
by inoculating the organisms mixed with mucin would prove of consider-
able value in establishing a new approach in the differentiation of the
two organisms, as well as in establishing the practical significance of
the subgrouping tendency of the swine organisms and in correlating cul-
tural, biologic, and epidemiologic characteristics of the organism.
D. CONCLUSIONS

1. *Hemophilus influenzae suis* organisms could be differentiated from *Hemophilus influenzae* organisms by carbohydrate fermentation reactions. The swine organisms fermented maltose and saccharose, the latter only slightly when a Seitz filter sterilized chocolate peptic digest-beef broth medium with Andrade's indicator was used. The majority of ten human strains fermented xylose, dextrose, and galactose, while, with the exception of one strain, they did not ferment maltose nor saccharose.

2. Indol was not produced by any of the swine strains, while 50 per cent. of the human strains were indol positive.

3. By agglutination and homologous agglutinin absorption tests the swine strains gave reactions differing from the human strains.

4. The swine strains had a tendency to fall in two subgroups when tested by cross-agglutination on the various homologously absorbed swine sera.

5. A distinct individuality in serologic behavior was noticed between the human strains, while the swine organisms, even though showing individual differences, appeared as a much more homogeneous group.
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PART II. ANTIBODY-RESPONSE TO EXPERIMENTAL SWINE INFLUENZA.

A. INTRODUCTION

Ever since Shope (9, 10) discovered the cause of swine influenza to be a filtrable agent in association with the organism Hemophilus influenzae suis, and since Smith, Andrewes, and Laidlaw (14) and Francis (2) described the human influenza virus as the etiologic agent of human influenza, a great deal of research has been done in order to establish stronger bases of study for the immunologic and epidemiologic characteristics of these two diseases and their etiologic agents.

In 1932, Shope (11), was able to demonstrate the production of an immunity and neutralizing antibodies towards the swine influenza virus following an attack of the disease, or after intramuscular inoculations of the living virus.

In 1934, Andrewes, Laidlaw, and Smith (1) and in 1935, Shope (12) were able to transmit the virus to the mouse. This finding proved to be a real asset in the study of immunity, as it represented a much more economical experimental animal than either the ferret or swine for the study of certain immuno-biologic phases of the two viruses.

Laidlaw, Smith, Andrewes, and Dunkin (8), in 1935, having prepared a hyper-immune horse serum to a human influenza strain, were the first to report on the use of the mouse in practical neutralization tests for the quantitative titration of influenza virus neutralization-antibodies.

Francis and Shope (3) and Shope (13) used a modification of this virus neutralization test in mice in their studies on convalescent and immune swine, ferret, and horse sera, in all of which they were able to find virus neutralizing antibodies, and especially toward the homologous virus strain.
One of the first reports on the immunologic behavior of the influenza viruses, as determined by the neutralization test on mice, was given by Smith, Andrews, and Laidlaw (15), in 1935, when they reported that immunity toward the human influenza virus in the ferret lasted for three months, while the neutralizing antibodies still remained as high as 1:100 to 1:2500.

In 1936, Smith and Stuart-Harris (16), reporting on the accidental infection of the latter with one of the ferret passages of the human virus "WS", found that the virus-neutralizing antibodies began to appear sometime after the third day, gaining their climax (1:50) between the sixteenth and thirty-first days, and showing a small and gradual decline on the forty-fourth and eighty-first days' bleedings. These findings were again reported by Smith (17) in 1937.

Francis and Magill (4) and Francis (5), in 1937, reported on the antibody response of a number of vaccinated persons, as well as that of three patients having developed a natural infection of influenza. These patients were shown to present an increase in antibodies from 0 or 1:5 to 1:40 and 1:120 respectively by the twenty-first day, followed by a slight decline on the one hundred and eightieth day. Similar changes were encountered with the vaccinated individuals, in which the antibody rise was approximately ten-fold and the increase was found to occur rather abruptly and during the second week, followed by a stationary period lasting for two months, and a decline in antibody titre by the fifth month.

Francis, Magill, Beck, and Riordan (6) reported on a sharp rise in antibodies after the recovery from the disease, with values of 1:26 for
the acute sera and of 1:210 for the convalescent sera. These authors found a rapid increase in antibody titre in one case by the seventh day, it then reached its highest level on the fourteenth day, and began showing a decline on the twenty-third day after infection.

The same authors (7) in a more complete study on the antibody response to human influenza, found that, whereas the antibody titres at the acute stage of the disease were on an average 1:21, those at convalescence were 1:305, which five months later had decreased almost 50 per cent. to a titre of 1:162. This decrease was found to be the smallest with titres lower than 1:100. These authors observed, also, an increase in circulating antibodies in the blood of contacts not having shown clinical evidence of the disease. These individuals presented much smaller amounts of antibody, showing an average of 1:60.

Smorodintseff, Tushinsky, Drobyashevskaya, Korovin, and Osetroff (18) reported 25 to 100-fold increases in the neutralising antibody titres of the sera of their human volunteers experimentally infected with the human influenza virus, when they were bled from ten to fifteen days after infection.

The presence of swine influenza virus neutralising-antibodies after recovery to swine influenza has been reported a number of times in the literature. The time of the appearance of antibodies, the time and degree of occurrence of maximum antibody titres, and the behavior of these titres during longer periods of time, in comparison to the reports on the human virus, had not been determined. The present experiments were, therefore, conducted in order to obtain this information.
B. EXPERIMENTAL

1. Materials and Procedure.

The swine in the experiment were obtained from disease-free stock raised in the experimental farm of the Animal Pathology Department of the Rockefeller Institute for Medical Research at Princeton, New Jersey. These animals were known not to be *H. influenzae* suis carriers. The animals were of various ages and of the Chester White breed. One of the animals, swine 2002, had been inoculated intracranially previously with material suspected of containing hog-cholera virus, but it did not develop the disease so it was considered safe to use it in this experiment.

The virus used was swine influenza strain 15, which had been isolated in Iowa in 1930 by Dr. Shope (10), while the *H. influenzae* suis cultures used included several cultures from different swine passages of strain 1916.

The mice used in the virus neutralization tests were of the Rockefeller Institute stock and were two to three weeks of age. These animals were known to be free of the chorio-meningitis virus.

Each of a series of five swine were infected intranasally with a mixture of swine influenza virus and *H. influenzae* suis. Previous to this the animals had been bled from the tail and normal control samples of serum obtained by centrifugation and by Seitz filter sterilization. The tubes were then plugged with sterile rubber stoppers and kept in the ice box until used.

The virus-organism mixture was prepared with 9 c.c. of a 5 to 10 per cent. suspension of glycerinated swine lungs containing swine influ-
ensa virus strain 15 and 1 c.c. of a pooled concentrated suspension of a 24 hour agar-horse-blood suspension of several H. influenzae suis strains. The mixture was prepared just before the introduction of 5 c.c. into each nostril of each of the five animals.

The sixth animal, swine 2002, received only virus 15. This was obtained from a 5 per cent. suspension of glycerinated mouse-lungs containing the virus. This animal was inoculated with an amount of virus similar to that received by the other animals.

These animals were then tail-bled at regular intervals through a period extending for 11, 31 and 84 days. The blood of these animals was allowed to coagulate and the serum extracted after its centrifugation. The serum was then sterilised by Seitz filtration and then kept in test tubes in an ice box.

Virus Neutralization Technique: The neutralization tests were conducted in the manner described by Francis and Shops (3), employing the supernatant of a 2 per cent. suspension of glycerinated infected mouse lungs as the virus source. These pieces of tissue previously had been washed three times in physiological salt solution and then ground in a sterile mortar with sand. The virus was then diluted in physiological salt solution making a 2 per cent. suspension and the supernatant fluid was mixed with the various dilutions of the sera which were to be tested. These dilutions were prepared using 0.2 c.c. of the original serum and varying amounts of physiological salt solution. The sera were then further diluted one half when mixed in equal amounts with 1 c.c. of the virus suspension. The mixtures were then kept in an ice box for two hours prior to their inoculation into mice. In each of the experiments the normal swine sera were tested undiluted as virus controls.
Three mice were used in each test. Each mouse was subjected to ether anesthesia, and then inoculated by dipping its nose in the serum-virus mixture in a slightly tilted small Petri dish. The mice, while under ether anesthesia, were then allowed to breathe for three to four seconds in the fluid and they were then placed in cages. As many as nine mice were placed in each cage in order to save space, for the virus is not contagious in mice.

The mice were then observed daily and those that died were placed in an ice box and an autopsy performed. Most of the animals died from the fourth to the seventh day, but the test was carried over a period of ten days, after which no deaths occurred. Those surviving this period were sacrificed and examined for the presence of influenza lesions.

At necropsy, the mice were graded as to the presence of influenza lesions as well as degree of pulmonary involvement, using the figures 1+ to 4+ to indicate the number of lobes involved. The mice which died during the ten-day period showed 4+ lesions, while those surviving showed either none or 1+ to 4+ lesions.

In the interpretation of results, the animals which succumbed during the ten-day observation period and showed a typical influenza pathology at autopsy were considered to have received a non-neutralizing dilution of the serum, whereas those which survived that period were considered to have received a neutralizing dilution of the serum. The final titre of a given serum was then taken as the highest dilution which protected all or the majority of the mice against death. In addition, the extent of lung involvement and, to a certain degree, the time of death were influential in the determination of the final antibody titres of the various sera.
2. Results.

Four of the six animals used in this experiment developed typical swine influenza characterized by temperatures of 40 to 41.6°C, the loss of appetite, depression, cough, and dyspnea, followed by a return to normal on the sixth to seventh day after the time of infection. The fifth animal developed a mild afebrile illness like the "filtrate disease", while the sixth, which was injected only with virus, developed the usual "filtrate disease" described by Shope (10).

The clinical and antibody behavior of the various animals was the following: (See Table I and Charts 1 - 6)

Swine 1984, see Chart 1, presented a characteristic swine influenza attack one day after inoculation. Its temperature curve reached a peak of 40.9°C. and it remained high while the clinical symptoms persisted, that is, up to the sixth day. At the same time, the presence of virus neutralizing antibodies was first noted on the seventh day, increasing to 1:40 on the fourteenth day, and reaching its peak at 1:120 on the twentieth day.

Swine 1985, see Chart 2, became characteristically ill with swine influenza on the first day, showing a high temperature reaction reaching 41.6°C. lasting for six days while clinical symptoms disappeared on the seventh day, simultaneous with the first appearance of antibodies. The latter increased rather abruptly, reaching a 1:80 concentration on the tenth day, and their peak, at 1:160, on the fifteenth day after infection.

Swine 1974, see Chart 3, developed a typical attack of swine influenza, reaching a temperature of 41.6°C. after three days. The fever disappeared in six days, while the clinical symptoms persisted until the

Days after inoculation

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Signs of swine influenza

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Days after inoculation

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Signs of swine influenza

+ + + + + +

Chart 1

Chart 2
Chart 3


Days after inoculation

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Normal temp.

Chart 4


Days after inoculation

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Normal temp.
Chart 5


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Signs of swine influenza: + + + + + + 

Titer of neutralizing antibodies

Chart 6


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Temperature

Normal temp.
seventh day, when the first neutralizing antibodies were noticed. The antibody response of this animal was relatively slight, reaching its maximum of only 1:60 on the fourteenth day, and showing a descent to 1:40 on the sixteenth day. The antibodies remained stationary at this level with an indication of a lowering in the titre on the eighty-fourth day.

Swine 1975, see Chart 4, which was injected with both virus and bacteria presented an unusual behavior; it showed no rise in temperature throughout the experiment and only the slightest symptoms of malaise and inappetence for two days. It did not show antibodies in its normal serum, and its virus neutralizing antibodies became evident for the first time sometime between the seventh and tenth days. The antibodies further behaved peculiarly in that they became stationary at a 1:60 concentration from the fourteenth to the twenty-first days, after which they increased up to 1:120. This level was maintained until the forty-sixth day, after which the antibody titres descended to 1:80.

Swine 1993, see Chart 5, while only studied for eleven days, showed a typical swine influenza illness with temperatures as high as 41°C for six days and with clinical symptoms up to the seventh day. In this animal the virus neutralizing antibodies appeared on the sixth day's bleeding and its peak was reached on the next day, attaining only a 1:20 concentration, which remained until the eleventh day, when the experiment was terminated.

Swine 2002, see Chart 6, was infected with virus alone and developed a characteristic attack of "filtrate disease". There was no significant elevation of temperature and clinically the illness was characterized by a malaise and inappetence of two days' duration. Neutralizing antibodies became first detectable sometime between the seventh and tenth days. The
antibody titre rose gradually up to 1:80, which occurred on the twenty-seventh day after infection.

Swine 1984 and 1985 were tested for active immunity to swine influenza on the twentieth day and found to be solidly immune. Serum drawn 6, 12, 24, 50, and 72 hours after the immunity test showed no significant changes in neutralizing antibody titres.
C. DISCUSSION

The results presented in Chart 7 illustrate the individuality in antibody response of different swine to swine influenza. The only close agreement was the time of the first appearance of neutralizing antibodies. Four of the swine tested showed the presence of antibodies on the sixth and seventh days after infection, whereas swine 1975 and 2002 did not show them until sometime between the seventh and tenth days.

The time required for the antibodies to reach their maximum titres and the height of these titres were highly individual. The maximum antibody titres observed, excluding those of swine 1993 that were studied for only eleven days, ranged between 1:60 and 1:160, and they were attained in from the fourteenth to the twenty-seventh days after infection. The latter occurred in swine 1975 and 2002. With these animals, it may be significant that, while they were the ones showing the slightest symptoms, they were also the ones showing a delayed production of antibodies, as shown by the later appearance of antibodies and their delayed reaching of maximum values.

In two animals kept under observation for eighty-four days there was evidence of a decrease in titre, especially in the serum having shown the higher neutralizing antibody titre, which has also been observed by Francis and his coworkers (7) in their investigations on human influenza convalescent sera.

In comparison to the human influenza virus reports given by Smith and Stuart-Harris (18), by Francis and his coworkers (4, 5, 6, 7) and Smorodintseff et al (18), it was found that both viruses behaved in a quite similar fashion, even though the maximal convalescent titres re-
Chart 7

Comparative antibody response of the six strains.
ported by Francis and his coworkers (7) were considerably higher, as they averaged 1:306, whereas those of the five swine sera reported in this experiment averaged only 1:108. Still, most of the human influenza cases showed acute or original antibody titres averaging 1:21, whereas the swine did not show virus neutralizing antibodies in their normal sera.

In the four swine which presented symptoms of swine influenza, the appearance of antibodies seemed to coincide with the disappearance of fever and other clinical symptoms, as well as with the onset of convalescence. This suggests that the presence of these antibodies may have contributed to the cessation of the signs of illness. This possibility is still further enhanced by the fact that it has been found that the virus of swine influenza disappears from the respiratory tract in seven or more days after infection, Shope*. The same has been reported by Francis and his coworkers (6) with human influenza. Still, these animals are supposed to continue showing anatomic-pathologic changes for a variable period of time after recovery has been apparently complete.

In swine 1975 the absence of the typical swine influenza symptomatology may be possibly explained by a failure of the H. influenzae suis organism to become established, allowing only the production of the "Filtrate type" of disease. Still, the occasional animals showing this type of a disease are said to have contained both the virus and the organism upon autopsy on the third or fourth days after infection, Shope*. On the other hand, the failure of the organism's establishment has been reported by Shope to occur when inoculating it accompanying the human influenza virus.

*Personal communications.
D. CONCLUSIONS

1. Swine influenza virus neutralizing antibodies made their first appearance in swine with clinical experimental swine influenza on the sixth and seventh days after infection, coinciding with the disappearance of fever and the other clinical symptoms.

2. In the two swine with the milder "filtrate disease" the swine influenza neutralizing antibodies did not appear until sometime between the seventh and tenth days.

3. The maximum antibody titres ranged from 1:60 to 1:160 and were attained from the fourteenth to the twenty-seventh days after infection.

4. A general tendency of decrease in neutralizing antibody titres was noted in two of the swine studied for eighty-four days.

5. The antibody response to swine influenza was observed to be similar to the literature reports on human influenza.
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