FURTHER OBSERVATIONS ON THE RELEVANCE OF SEROLOGIC RECAPITULATIONS OF HUMAN INFECTION WITH INFLUENZA VIRUSES*

By F. M. DAVENPORT, M.D., A. V. HENNESSY, M.D., J. DRESCHER, M.D., J. MULDER, M.D., AND T. FRANCIS, JR., M.D.

(From the Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor; Robert Koch Institute, West Berlin, Germany; and University Hospital, Department of Internal Medicine, and the Institute of Preventive Medicine, Leiden, Netherlands)

(Received for publication, July 20, 1964)

The results of studies on the age distribution of antibodies, on the range of antibody response to monovalent vaccines by age, and on the age-specific characteristics of antibody demonstrated by serum adsorption tests, have indicated that periods of past prevalence of strains closely related antigenically to swine and Asian strains can be identified. Thus a swine-like virus is thought to have been involved in the pandemic of 1918 and an Asian-like one in the pandemic of 1889–90.

A basic tenet of these serologic recapitulations is that the major antigens of the initial infections of childhood orient antibody formation throughout the rest of life. Upon subsequent exposures to antigenically different yet related strains, the level of the primary antibody is reinforced (1–9).

The relevance of this thesis has been questioned by others who take the position that the presence of age-specific antibody patterns cannot be used as a basis for identification of the primary infections because after repeated experiences, minor antigenic components of infecting strains may induce antibody which reacts with the major antigens of viruses to which the subject has not been exposed (10–13). Such an alternate explanation for the serologic findings in humans discounts the fact that titers to other isolates are generally lower than those to the age-specific strains of first infection (1, 2, 6).

It also ignores the temporal association between antibody patterns found in two younger cohorts of the population and the known periods of prevalence of influenza A and influenza A–prime (1, 2), and finally does not consider the fact that with the passage of time the basic antibody reactivity of the respective cohorts persists despite further natural exposures (1, 2, 9).

* This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, United States Army, Washington, D. C.

Recently acquired experience with a unique procedure for titrating antibody which can distinguish between reactions with antibody oriented to the major antigens of strains responsible for primary exposure and those encountered as minor antigens in repeated infections prompted a reexamination of the sets of sera utilized formerly for describing the age incidence of swine and Asian antibodies. The results of retesting these materials with this new technique comprise the substance of the present report.

Materials and Methods

Viruses.—The strains utilized were identified in each of the three laboratories collaborating in this report, and are described by the customary nomenclature currently used in each laboratory.

Sera.—(*a*) *Ferret:* Convalescent specimens were obtained from animals infected intranasally. Samples were drawn between 14 and 21 days after inoculation.

(*b*) *Preepidemic single specimens from man:* Those reported upon from the Netherlands were obtained before July 1957 (5). Those collected in Ann Arbor, Michigan, date from June to July, 1957, and were obtained from the same source as the preepidemic pools.

(*c*) *Preepidemic pools:* Serum specimens were drawn from patients attending the University of Michigan Hospital, Ann Arbor, during June and July, 1957. 25 aliquots were combined by the age intervals specified.

(*d*) *Postepidemic single specimens from man:* The 1962 Netherlands sera were collected at the Institutes of Health, Utrecht. All collections were made during interepidemic periods.

(*e*) *Treatment of sera:* Sera were treated with RDE (14) or periodate (15) to remove nonspecific inhibitors.

HI Titrations.—Determinations were carried out by a standard pattern method with four units of virus and 0.5 per cent erythrocytes (16).

Photometric Titrations.—A complete description of the unique methods employed for measuring specifically reacting antigens or antibodies has been published (17, 18). Their advantages pertinent to this study are referred to where appropriate.

EXPERIMENTAL

Recognition of Specificity of Antibodies.—The photometric method employed in the present studies for measurement of antibody objectively discriminates between homologous and heterologous reactions. The basis for the distinction is that while an adaptation of Freundlich's equation ($\log Cbv/Cab = \log a + 1/N \log Cfv$) may be used to describe the proportionality between concentrations of free virus (*Cfv*), of bound virus (*Cbv*), and of antibody (*Cab*) operative in a homologous system over a wide range, with heterologous components the equation cannot be used since a different type of proportionality pertains.

One consequence of this fact is that as the photometric method is currently used, it is impossible to assign an antibody titer in Antibody Concentration Units (ACU) to a serum specimen reacted with a heterologous virus. Hence the symbol "—" used in tables of this report does not imply that no inhibition of hemagglutination was observed, but rather that the reaction, if any, was of

the heterologous variety. Conversely all titers given in ACU imply that the reaction was specifically oriented to the strain used for testing, and the criterion of specificity is that the data obtained fit those predictable from Freundlich's equation (18).

A demonstration of the discriminating power of this photometric method is given in Table I. Convalescent ferret sera containing hemagglutination-inhibiting antibodies reacting with swine virus were prepared under different conditions. In the first case the animals were sequentially inoculated with A and A-prime strains, but did not receive swine strain (ferrets 1 to 3). In the second, the ferret was infected once with Shope strain 15 (ferret 4). The titer values shown in parenthesis after the designation of each strain used for infection were determined simultaneously in Leiden for each serum set. The final

TABLE I

Photometric Recognition of Specific Antibody in Ferret Sera

Ferret No.	Sequence of infections and hemagglutination inhibition titers against swine strain after each exposure	ACU titer of final specimen against swine strain
1	A/PR8 (<9), A/PR8 (<9), A_1/1947 (120), A_1/1951 (250), A_1/1951 (1050), A_1/1953 (1050), and A_1/1956 (1900)	—
2	A/WS (<9), A/WS (<9), A/PR8 (100), and A_1/1947 (450)	—
3	A/WS (70), A/WS (100), A/PR8 (1450), A_1/1947 (800), A_1/1949 (400), A_1/1951 (400), A/1953 (300), and A_1/1956 (1150)	—
4	Swine strain 15 (3000)	9414

— = <12.5 ACU or heterologous reaction.

serum of each set was then sent to Berlin under code and tested photometrically for specific antibody. Even though the first three final specimens contained high levels of cross-reacting swine antibody, when tested by hemagglutination inhibition, they did not exhibit strain-specific antibody when tested photometrically. The lowest dilution of serum used was capable of detecting as little as 12.5 ACU. The fourth specimen was found to contain a high titer of specifically reacting swine antibody. The data clearly demonstrate that this photometric procedure is capable of differentiating between the dominant strain antibody and that stimulated by minor antigens.

The Specificity of Antibodies to Swine Strain found in Selected Specimens of Human Serum.—It was previously reported that upon examination of materials collected in 1952, antibody against swine virus was not detected by hemagglutination inhibition in pools of serum representing persons less than 29 to 30 years of age. In pools representing persons from 29 to more than 65 years of age, antibody reacting with swine virus was consistently found (1, 2). When individual serum specimens obtained in 1953 and in 1957–58 were studied

(19, 6), a relatively small number of individuals in the first two decades of life were found to possess antibody reacting with swine virus, and in the third there was a moderate increase in the frequency of occurrence of these antibodies. Apparently the existence of those individuals who atypically possessed swine antibodies had been obscured by dilution as the pools were made. At the time the data first became available, the presence of hemagglutination-inhibiting antibodies to swine strain in the sera of persons less than 30 years of age was attributed either to (*a*) cross-inhibition from the high levels of A or A-prime antibodies characteristically found in the sera of such persons or (*b*) to a persistence of swine-like antigens as major components of viruses sporadically appearing after the principle period of prevalence of swine-like strains (19).

An opportunity to acquire information which might help to differentiate between the two possibilities came when arrangements were made to compare

TABLE II

Antibody Titers Against Swine Strain Found in Single Specimens of Coded Sera Collected in the Netherlands in 1962

Age	22	23	24	25	30	32	33	34	34	36	38	40	40	42	42	44	46	48	48	50	50	52	52	79	80
HI titer	100	45	90	30	100	110	110	400	50	100	180	280	800	200	1600	1600	2300	3650	1800	400	200	400	800	2300	1150
ACU titer	—	—	—	—	—	—	—	—	—	—	—	—	1665	1215	—	—	2226	8028	—	—	—	3072	6130	7579	—

— = <12.5 ACU or heterologous reaction.

ACU and hemagglutination inhibition antibody titers in individual serum specimens collected in the Netherlands in 1962. The age range was 22 to 80 years and each had been shown in Leiden to contain antibody to swine strain as measured by hemagglutination inhibition. They were coded to conceal age and sent to Berlin for further testing. Table II records the age of the donors and the swine antibody levels found in each serum sample photometrically and by hemagglutination inhibition.

It is evident that application of this photometric method distinguishes between the hemagglutination-inhibiting swine antibodies found in the sera of persons less than 40 years of age from those found in persons 40 or more years old. None of the specimens from the former group contained antibody which yielded a specific swine ACU titer, while 7 of the 14 specimens in the later age range exhibited antibody which reacted specifically with swine virus.

The findings are interpreted to indicate that the presence of swine antibody measured by conventional hemagglutination inhibition methods in serum specimens obtained from persons resident in the Netherlands who were less than 40 years of age in 1962, is attributable to cross-reactivity with antibodies induced by exposure to strains other than swine. Conversely one-half of the

persons tested who were aged 40 or more, showed evidence of prior strain-specific exposure to swine virus. The resolving power of the photometric method employed is once again illustrated. From these limited data, it would appear that the disappearance of swine-like strains from circulation in humans was fairly abrupt, occurring possibly in or about 1922, and that the hypothesis of sporadic recurrence of strains containing swine-like antigens as major antigenic components is not supported.

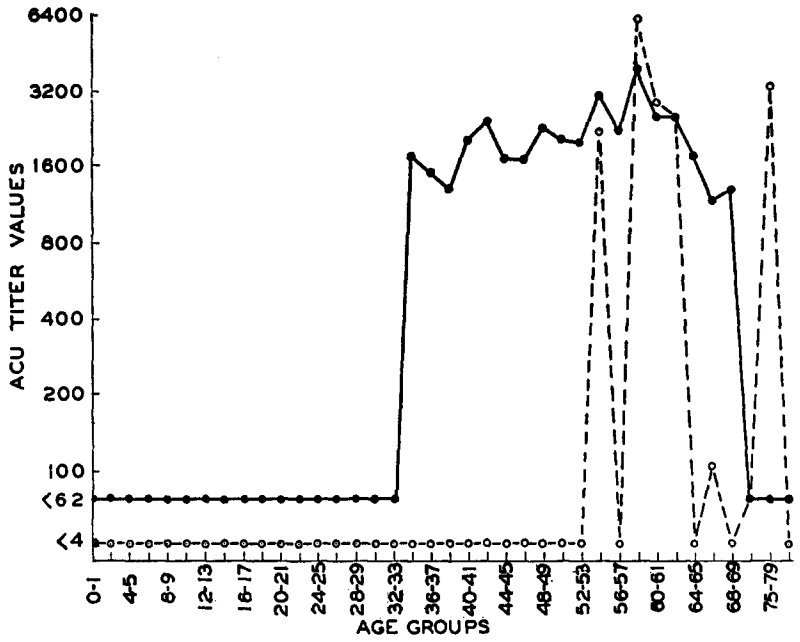

FIG. 1. Age determination of swine and Asian antibodies determined photometrically.

Age Distribution of Specifically Reacting Swine Antibody found in Pools of Serum Collected in Michigan in the Summer of 1957.—Confirmation of the indication that the period of prevalence of swine-like strains ceased rather abruptly in the early 1920's, and further information concerning the relevance of the distribution of swine antibodies by age, was sought by testing photometrically the content of swine antibodies present in pools of serum collected for other purposes before the advent of Asian influenza in 1957.

The findings are reproduced in Fig. 1. Specific antibody was not detected in pools of serum representing persons less than 34 years of age, when the test was conducted with dilutions capable of measuring as little as 62 ACU of swine antibody. In contrast pools representing persons from 34 to 69 years of age showed consistent and most often high levels of specifically reacting swine anti-

body. Swine antibody was not detected in pools collected from persons 70 years of age or more. However, a relatively low frequency of ACU swine titers was found in individual specimens collected between 1957 and 1962 from persons in their 70's or 80's. Masurel and Mulder have independently shown that the frequency of swine antibodies declines in the late decades of life (6).

The age distribution of specifically reacting swine antibody found in the 1957 collection is remarkable in two respects. Firstly, the concurrent change with the passage of time in the age of onset of swine antibodies measured by several different methods in studies carried out in several different years, is striking. Thus, in specimens collected in 1935, little neutralizing swine antibody was found before 10 years of age (20). Employing specimens collected in 1952, the age of onset of swine antibody detected in pooled sera by conventional hemagglutination inhibition was 29 to 30. In pools made 5 years later, the age of specific swine antibody measured in ACU had shifted to age 34 to 35. When individual sera collected in 1962 were tested photometrically for specific antibody the age of onset was 40. Clearly this cohort, as was previously stated (1, 2, 9), is advancing through life carrying a unique antigenic imprint which past experiences with swine-like strains have made indelible. The separate studies identified 1922, 1923, or 1925 as the year of birth of the youngest members of this unique group.

Secondly, the lower frequency of swine antibodies in serum specimens collected from the oldest age groups is incompatible with the thesis that the presence of swine antibodies in persons aged 34 to 69 is due to repeated stimulation by strains carrying minor amounts of swine antigen. If that were the case swine antibodies would be found consistently in the sera of senior citizens.

Identification of Specifically Reacting Asian Antibody in Sera Collected before Asian Influenza became Epidemic.—The significance of the presence of Asian antibodies in the 1957 pre-epidemic collections of human sera have been evaluated in two different ways. One group reported that Asian antibodies measured by pattern tests were peculiarly frequent in specimens collected from persons in the seventh or older decades of life, and interpreted these findings to indicate that Asian-like strains had had a period of past prevalence which encompassed the pandemic of 1889–90 (5, 7, 8). Another did not observe a disproportionate increase in the frequency of Asian antibodies in the sera of older persons and attributed the irregular results obtained to cross-reactivity with antibodies fundamentally oriented against other strains (11–13, 21).

Since it was realized that the discordant results and interpretations could not be resolved by further consideration of the limited data available, it was felt important to obtain additional information by ascertaining the strain-specific reactivity, in ACU, of the remaining samples of former serum collections.

The first step was a reexamination of the serum pools collected in 1957 at Ann Arbor prior to the appearance of Asian influenza. Only seven pools of

serum were found to contain ACU antibody titers reacting specifically with the A_2/AA/23/57 strain (Fig. 1). Thus at 54 to 55 years, the ACU titer was 2369; at 58 to 59, 6301; at 60 to 61, 2947; at 62 to 63, 2674; at 66 to 67, 108; at 70 to 74, 65; and at 75 to 79, 3432. All other pools representing persons from less than 1 to 84 years of age were negative for specifically reacting antibody at dilutions of serum which in general were capable of detecting specifically reacting antibody if present at a concentration as low as four ACU.

Additional information came from studies carried out in Berlin, utilizing a set of coded sera sent from Leiden. Samples from persons aged 42 to 90, whose sera, when tested in Leiden by hemagglutination inhibition and mouse protection tests, yielded positive or negative results (5), were included in the set. The code disguised the age of the individual and his serologic status as determined in Leiden. Each specimen was tested for Asian antibody reacting specifically in the photometric test with either A_2/AA/23/57 or A_2/Japan/305/57 strains.

TABLE III

Antibody Titers Against Asian Strains Found in Single Specimens of Coded Sera Collected in the Netherlands before July 1957

Age	42	52	59	59	61	61	62	64	71	72	72	78	76	80	81	82	82	83	85	85	85	88	88	90
HI titer	36	45	72	<9	<9	<9	18	37	87	18	54	103	13	204	150	129	672	40	102	<9	<9	<9	<9	<9
ACU titer	908	146*	—	165*	—	—	975	751*	1675	—	166*	134	—	858	634	340	505	—	238*	—	—	—	—	—

— = <18.5 ACU or heterologous reaction.

* Titrated with A_2/Japan/305/57; negative to A_2/AA/23/57.

The latter was included in the experiment because it has been found that antibody to these two 1957 isolates can be differentiated photometrically.

The results of the comparison are shown in Table III. There was a marked correlation between the findings obtained photometrically and those obtained by hemagglutination inhibition. Agreement was the case in 19 of the 24 samples.

Finally a set of individual sera from the preepidemic 1957 Ann Arbor collection were tested there for Asian antibody specifically reacting in the photometric test with the A_2/AA/23/57 strain. 61 specimens were examined, each of which had exhibited antibody measured by hemagglutination inhibition with the A_2/AA/23/57 isolate. None had been screened by mouse protection tests. The findings are summarized in Table IV. The sensitivity of the test was 12.5 ACU.

Specifically reacting antibody was not found photometrically in any of the hemagglutination inhibition–positive samples obtained from persons 7 to 54 years of age. Three of 7 persons aged 55 to 65 years, and 7 of 13 persons aged 68 to 77 years exhibited specifically reacting Asian antibody. Neither of the two persons 82 years of age possessed a specific Asian ACU titer. In passing, it is of interest to speculate that the closer agreement between the hemaggluti-

nation inhibition and ACU values found in the individual sera examined from Leiden compared to those examined from Ann Arbor may be related to the fact that the former set was pretreated with RDE.

These data cannot be considered to represent precise information about the pre-1957 pandemic age-specific incidence of Asian antibodies since the specimens examined were not randomly selected. Nevertheless from the total findings, two conclusions seem warranted. The first is that specifically reacting antibody is not frequently found in the sera of persons less than 55 years of age in 1957. The second is that from age 55 to about 79, the occurrence of specifically reacting Asian antibody is peculiarly high.

Taken together the composite data on the age distribution of specifically reacting Asian antibodies strongly support the interpretation previously proffered that the Asian strains prevalent in 1957 and since are not a totally *newe*

TABLE IV

Presence of Asian Antibody in a Selected Set of Sera Collected in Ann Arbor before the Asian Epidemic of 1957

Age group	No. of sera	No. giving positive ACU test (with A_2/AA/23/57)
yrs.		
7–54	38	0
55–65	7	3
68–77	13	7
82	2	0

acquayantance antigenically, but are either identical with or closely related antigenically to strains of virus prevalent formerly.

DISCUSSION

The practice of serologic archeology is a hazardous venture similar to the challenge of being obligated to attempt the reconstruction of the skeleton of a dinosaur from a few teeth and some fragments of bone (22). Standing by themselves without reference to the sum total of accumulated information, the reconstructions presented seem at first grotesque. Moreover, unless constantly reexamined as new information becomes available, the models may persist either as unacceptable factitious reconstructions or justifiable credence concerning the validity of the model may be withheld. The present investigations were deliberately undertaken to test the relevance, by application of a new technique, of the hypothesis that the age of appearance and the period of high prevalence of antibodies oriented against two prototype strains of influenza A provide a serologic recapitulation of the past experience of two cohorts of the population with swine and Asian strains. The new information adds additional important support to the basic thesis.

The use of a photometric method which has a high resolving power for distinguishing between homologous and cross-reacting antibodies has contributed data which sharpen the delineation of the unique age segment of the population which carries specifically reacting swine antibodies. The data are incompatible with the thesis that this cohort acquired these antibodies by repeated exposures to strains bearing minor amounts of swine-like antigen (10, 11, 13). Rather they support the conclusion that swine-like strains were prevalent during a former period which encompassed the 1918 pandemic episode and ended on or about 1922.

The new information on the age distribution of specifically reacting antibody to Asian strains is also compatible with the existence of a former period of prevalence of Asian-like viruses. Here the focus is somewhat blurred and the delineation of the dates of that epoch is more obscure. Contributing to the fact that the Asian reconstruction is more difficult is the circumstance that the number of sera available for study which were collected before the pandemic of 1957 is relatively small, and because of technical problems the analysis by hemagglutination inhibition has been hampered. Nevertheless, since the youngest persons found to have specifically reacting Asian antibodies were 55 years old or more in 1957, while the oldest was 85, the data support the thesis that Asian-like viruses were quite likely involved in the pandemic of 1889–90 and may perhaps have circulated to a limited extent until 1902 (5, 7, 8).

It is not clear why the frequency of Asian antibodies found in the Asian cohort is lower than that of swine antibodies in the swine cohort. One factor may be that swine isolates are more homogeneous antigenically than are Asian strains and if one were to employ a larger battery of Asian strains in the test, higher frequencies might possibly be found. The results shown in Table III of the present report suggest that possibility. Another factor may be that since the A_2 strains are antigenically quite removed from A, A–prime, and swine strains, the "booster phenomenon" may not operate at peak efficiency. Finally, the low avidity of A_2 strains for antibody might prohibit detection of very low titers. These possibilities are presented to point out that there is awareness of the lacunae which exist in the reconstructed model, which can only be filled by further investigation.

SUMMARY AND CONCLUSIONS

By use of a photometric method which objectively distinguishes between homologous and heterologous antibody reactions, the age distribution of specifically reacting swine and Asian antibodies present in sera of persons not recently exposed to either virus was established. The findings support the thesis that the unique distribution by age of swine and Asian antibodies recognizes two past periods of prevalence of swine and Asian-like viruses, respectively. The findings apparently refute the conclusion that the existence of age-specific Asian and swine antibody patterns is primarily attributable to the undirected or

passive accumulation of experience with Asian or swine antigens present as minor antigenic components in most strains of influenza A.

BIBLIOGRAPHY

1. Francis, T., Jr., Davenport, F. M., and Hennessy, A. V., A serologic recapitulation of human infection with different strains of influenza virus, *Tr. Assn. Am. Physn.*, 1953, **66,** 231.
2. Davenport, F. M., Hennessy, A. V., and Francis, T., Jr., Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus, *J. Exp. Med.*, 1953, **98,** 641.
3. Davenport, F. M., and Hennessy, A. V., A serologic recapitulation of past experiences with influenza A; antibody response to monovalent vaccine, *J. Exp. Med.*, 1956, **104,** 85.
4. Jensen, K. E., Davenport, F. M., Hennessy, A. V., and Francis, T., Jr., Characteristics of influenza antibodies by serum adsorption, *J. Exp. Med.*, 1956, **104,** 199.
5. Mulder, J., and Masurel, N., Pre-epidemic antibody against 1957 strain of asiatic influenza in serum of older people living in the Netherlands, *Lancet*, 1958, **1,** 810.
6. Masurel, N., and Mulder, J., Studies on the content of haemagglutination inhibiting antibody for swine influenza virus A, *Verhandl. Het Nederlands Inst. Praev. Geneesk.*, 1962, **52,** 1.
7. Davoli, R., and Corsi, O. B., Anticorpi verso virus dell'influenza A-Asia neua popolazione normale, *Sperimentale*, 1957, **107,** 358.
8. Davenport, F. M., and Hennessy, A. V., The clinical epidemiology of Asian influenza, *Ann. Int. Med.* 1958, **49,** 493.
9. Francis, T., Jr., On the doctrine of original antigenic sin, *Proc. Am. Phil. Soc.*, 1960, **104,** 572.
10. Isaacs, A., Antibodies to influenza viruses in the sera of Nigerians, *J. Hyg.*, 1957, **55,** 485.
11. Henle, W., Lief, F. S., and Fabiyi, A., Strain specific complement-fixation test in antigenic analysis and serodiagnosis of influenza, *Lancet*, 1958, **1,** 818.
12. Hilleman, M. R., Flatley, F. J., Anderson, S. A., Luecking, M. L., and Levinson, D. J., Distribution and significance of asian and other influenza antibodies in the human population, *New England J. Med.*, 1958, **258,** 969.
13. Lief, F. S., and Henle, W., Antigenic analysis of influenza viruses by complement fixation. VI. Implications of age distribution of antibodies to heterologous strains following infection and vaccination, *J. Immunol.*, 1960, **85,** 494.
14. Brans, L. M., Hertzberger, E., and Brinkhorst, J. L., Studies on the elimination of non-specific inhibitors in sera against influenza viruses with the aid of filtrates of Vibrio cholerae, Antonie Van Leeuwenhoek, *J. Microbiol., and Serol.*, 1953, **19,** 309.
15. Communicable Disease Center Surveillance Bulletin No. 38, March 19th, 1958.
16. Committee on Standard Serologic Procedures in Influenza Studies, An agglutination inhibition test produced as a standard reference in influenza diagnostic studies, *J. Immunol.*, 1950, **65,** 347.

17. Drescher, J., Hennessy, A. V., and Davenport, F. M., Photometric methods for the measurement of hemagglutinating viruses and antibody. I. Further experience with a novel photometric method for measuring hemagglutinins, *J. Immunol.*, 1962, **89**, 794.
18. Drescher, J., Davenport, F. M., and Hennessy, A. V., Photometric methods for the measurement of hemagglutinating viruses and antibody. II. Further experience with antibody determinations and the description of a technique for analysis of viral mixtures, *J. Immunol.*, 1962, **89**, 805.
19. Hennessy, A. V., Davenport, F. M., and Francis, T., Jr., Studies of antibodies to strains of influenza virus in persons of different ages in sera collected in a post-epidemic period, *J. Immunol.*, 1955, **75**, 401.
20. Shope, R. E., The incidence of neutralizing antibodies for swine influenza virus in the sera of human beings of different ages, *J. Exp. Med.*, 1936, **63**, 655.
21. Clarke, S. K. R., Heath, R. B., Sutton, R. N. P., and Stuart-Harris, C. H., Serologic studies with asian strain of influenza A, *Lancet*, 1958, **1**, 814.
22. Mulder, J., Virology of pandemic influenza. Facts and speculations, Boerhaave Conference on Respiratory Virus Diseases, Leiden, Holland, April 1962, in press.