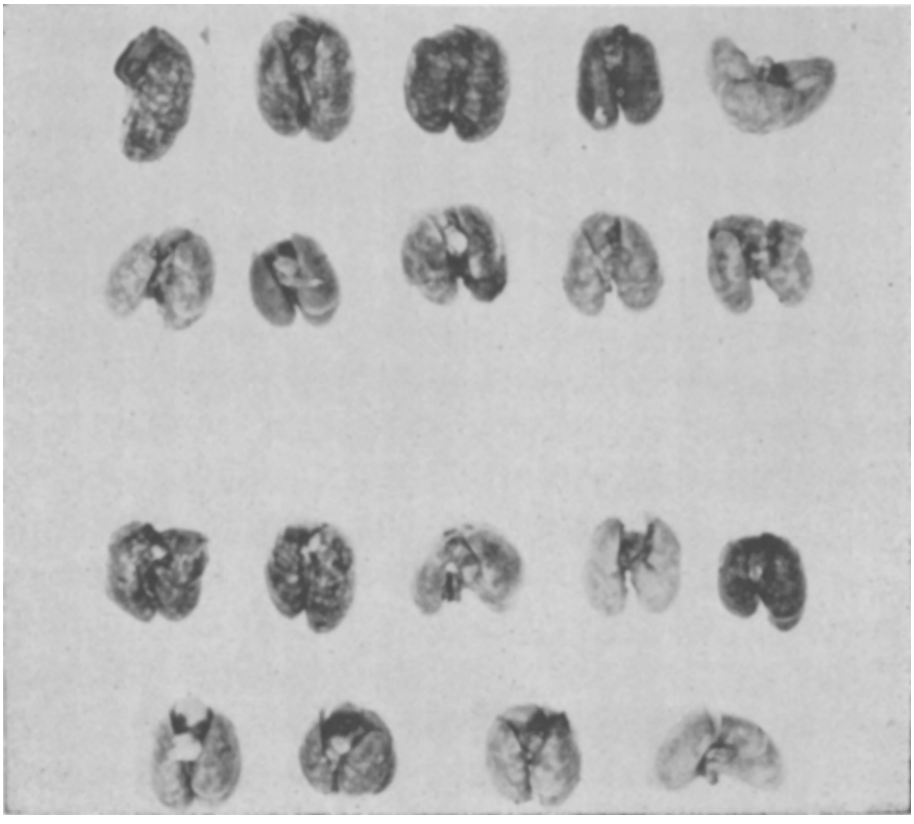

FIG. 4.
Lungs of mice infected with streptomycin resistant strain 67. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

sensitivity of these reisolated cultures was found to be the same as before injection into the mice.

Summary. Streptomycin resistant human type tubercle bacilli were found to be as virulent for white mice as streptomycin sensitive strains. Infection produced in mice with these streptomycin resistant cultures was not suppressed by treatment of the mice with streptomycin.

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Variation in Influenza Viruses. A Study of Heat Stability of the Red Cell Agglutinating Factor.*

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For estimating the degree of stability of a variety of materials, both biological and non-biological, it is common practice to utilize elevated temperatures in order to accelerate

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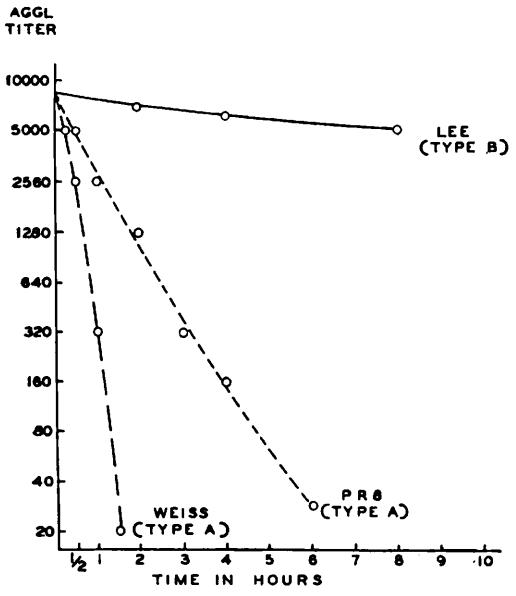

FIG. 1.

Curves showing comparative rates of inactivation at 61.5°C of Lee, PR8, and Weiss strains of influenza virus in untreated allantoic fluids.

the processes that occur slowly at normal temperatures. In view of the slow deterioration at temperatures up to 37°C of the immunizing and hemagglutinating antigens of influenza virus,[1-5] it seemed desirable to define a convenient temperature at which observations could be made rapidly in order to facilitate studies of the relative stability of different preparations of virus or vaccine. In the course of preliminary experiments at 56-65°C it was found that distinct differences exist among strains in terms of the rate of destruction of the red cell agglutinating capacity of the virus. These findings were explored further. It is the purpose of this preliminary communication to present the evidence obtained thus far revealing the existence of wide differences in the stability of hemagglutinin not only among strains but among different lines of the same strain as well.

Materials. Various strains were compared and in some instances the same strain which had been passaged in different hosts, or culture media, was examined. The strains and passage histories will be indicated in the text. The studies reported here have been done on allantoic fluid obtained without red blood cells from infected chick embryos.

Methods. As the source of heat, a water bath was used in which the temperature was accurately controlled within less than 0.1° in the region of 61.5°C This temperature was employed simply because the mercury thermo-regulator was originally set at this temperature when an attempt was made to adjust it to 62°C. Preliminary tests indicated that 62°C was convenient for the majority of strains examined. In some experiments at lower temperatures less well controlled heating equipment was used. For determining stability to heat, the virus suspensions were distributed in 0.5 cc quantities in small rubber-stoppered test tubes and placed in a rack suspended in the bath. At intervals, a tube containing the 0.5 cc aliquot was removed and immersed in iced water. All samples of the same virus suspension were stored in iced water or in the 4°C refrigerator and tested for titer of hemagglutinin at the same time.

The method for estimating hemagglutinating potency has been described.[6] The technic employed involves the use of 0.5 cc quantities of the serially diluted test material and an equal volume of 0.25% suspension of chicken erythrocytes. Serial dilutions were made with an automatic syringe. The endpoint of the titrations is taken to be the highest dilution showing complete agglutination as revealed by the pattern of the sedimented cells. Titers are expressed as the reciprocal of the final dilution of starting material. Procedures providing greater accuracy were not required since gross changes, rather than slight variations, were expected.

Results. The initial observations were made in the course of experiments with the 3 strains of influenza virus which were present in the vaccine recently used by the Army,[2] *i.e.*, the PR8 and Weiss strains of Type A virus and

1 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

2 Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

3 Salk, J. E., Pearson, H. E., Brown, P. N., Smythe, C. J., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 307.

4 Miller, G. L., *J. Exp. Med.*, 1944, **80**, 507.

5 Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

6 Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

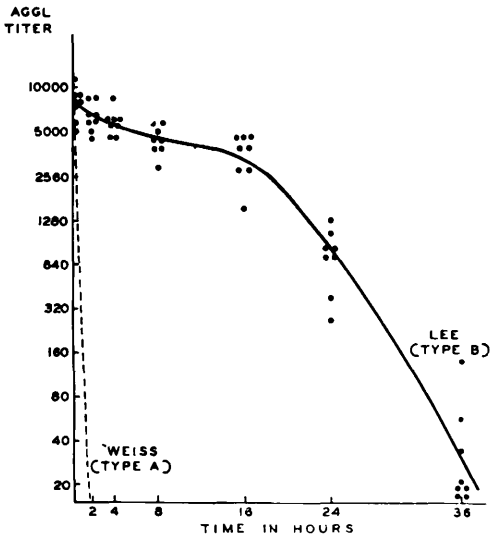

FIG. 2.

Curves showing rates of inactivation at 61.5°C of hemagglutinin of Lee strain of influenza virus Type B in 8 different samples of allantoic fluid and of Weiss strain of Type A virus in 12 different samples of allantoic fluid. (Degree of variation in 8 batches shown on Lee curve. Similar degree of variation along Weiss curve not shown.)

the Lee strain of Type B virus. The PR8 strain had previously been passed through a small number of ferrets, then through 593 mouse transfers, from which it was then introduced into the chick embryo for almost 50 passages. The Weiss strain had come through 3 ferret passages, 32 mouse transfers and 53 egg passages. The Lee strain, after about 8 ferret passages followed by 137 mouse passages, was in the 85th to 90th egg transfer. Fig. 1 shows the results of a typical experiment in which it is seen that the hemagglutinin of the Lee strain was much more stable than those of the PR8 and Weiss strains, and that Weiss was much less stable than PR8. That these variations are related to the different strains of virus and not to individual variations among different preparations of allantoic fluid is suggested by the data shown in Fig. 2. In this experiment 8 different pools of allantoic fluid containing the Lee strain and 12 different pools containing the Weiss strain were compared. While all 12 samples of the Weiss strain had no detectable hemagglutinin after 90 minutes at 61.5°C, little or no change in titer was observed among the 8 samples of the Lee strain in 2 hours. In fact, hemagglutinin of the Lee strain was still detectable after 24 hours, and in some samples after 36 hours.

The difference between the strains illustrated has been observed repeatedly and consistently with successive passages of these particular lines of the respective strains. That the differences are due to properties of the virus and not to some extraneous factor in the allantoic fluid is indicated by examination of the rate of inactivation of the hemagglutinin present in a mixture of the Weiss and Lee strains in allantoic fluid. The admixture with allantoic fluid containing the Weiss strain did not alter the shape of the curve of inactivation of the hemagglutinin of the Lee component. The difference between strains was still evident in preparations in which virus was separated from allantoic fluid by adsorption on red cells and elution[1,7] into a medium other than allantoic fluid, or after adsorption on calcium phosphate[8] and resuspension in physiological solutions. The influence of the composition of the physiological solution on stability will be described elsewhere.

In view of the striking difference in behavior of the Lee strain of Type B virus when compared with the PR8 and Weiss strains of Type A virus, it seemed of interest to determine whether the greater stability of the hemagglutinin was a characteristic of Type B strains. Accordingly, a variety of both A and B strains in allantoic fluid was examined.

A group of 8 Type A strains from different epidemics of the past several years was tested. The prior history of laboratory passage varied, but in all instances the strains exhibited less stability than the PR8 and Weiss strains. Since 61.5°C seemed to be above the critical zone of destruction of the hemagglutinin of these strains, preliminary tests were conducted at 50°C. At this temperature strain difference was evident.

Only 1 of 19 Type B strains tested exhibited a stability curve approximating that of the

[7] Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

[8] Salk, J. E., *Science*, 1945, **101**, 122.

Lee strain. The exception was the "BON" strain isolated in Dr. Burnet's laboratory in Australia in 1943.[9] Of the other 18 strains, all of which had been isolated in chick embryos in this laboratory, two† were obtained from a localized outbreak in May 1945 and the remainder† during the epidemic of 1945-46.[10] The 2 strains from the May 1945 outbreak had been passed 30 times in eggs and all of the 16 strains from the 1945-46 epidemic had been passed less than 10 times. Strain difference was again encountered. The hemagglutinin of all but 3 strains was destroyed in 5 to 15 minutes at 61.5°C. The 3 exceptions (Allen, Goodloe and Hacker), which were more resistant, were among the 16 strains obtained in the 1945-46 epidemic. Although the 2 strains isolated from the May 1945 outbreak had been passed 30 times in eggs, they were no more stable than the majority of the newer strains which had had fewer passages. It would appear from these observations that the particular line of the Lee strain, the stability of which has been described, cannot be considered representative of all Type B strains. Moreover, differences exist in the stability of the hemagglutinating property of strains of virus isolated from the same outbreak.

Since the Lee, PR8 and Weiss strains have had long lines of laboratory passage, it seemed possible that the difference between these and the recent strains might be related to this factor, in part at least. Accordingly, the Lee and PR8 strains of virus which had been maintained in different hosts for varying numbers of passages were transferred to eggs and the allantoic fluids obtained were then tested for stability of their hemagglutinating capacity. Tables I and II show the results of 2 such experiments. From these data it is seen that differences exist in the degree of stability of the hemagglutinin of the different lines of the same strain. In view of the greater stability of preparations having the longest history of egg passage, regardless of previous passage in other media, the following was done to determine the influence of change in host on the stability of the hemagglutinating property of the viruses. The regular egg-passage lines of the PR8 and Lee strains were transferred to mice and carried through 14 passages. After successive passages in mice the virus was returned to the egg and tests were made of the stability of hemagglutinin in the initial egg transfer from mouse material. In the series thus far, passage of the egg-line in mice has not altered the stability of the hemagglutinin of the respective strains of virus. The reverse of this experiment, *i.e.*, passage of the ferret or mouse lines through 27 egg transfers, has not changed the character of the virus in terms of the heat stability of the hemagglutinin.

The persistence of line difference, even after change in host or culture medium, suggests that the host factor is probably of secondary importance and that intrinsic differences of an inheritable nature are involved. The following experiment is cited in further support of the suggestion that the viruses maintained in the separate lines are distinct in terms of heat stability of the hemagglutinin. Chick embryos were inoculated with mixtures of the standard egg-line (Table II, Col. 1) and the ferret egg-line (Table II, Col. 3), combined in varied proportions. Examination of the infected allantoic fluids for heat stability of hemagglutinin indicates that viruses of both lines grew simultaneously and were present in different concentrations depending upon the relative quantities of the respective lines of virus in the different inocula.

Reference has been made to results of other studies that have a bearing on the observations reported here. In Hirst's studies,[1] in which he found that infectivity was destroyed more rapidly than hemagglutinating activity, the PR8 and Lee strains did not differ in the rate at which hemagglutinin was destroyed at 55 and 60°C. Miller[4] followed the rate of decline of infectivity and hemagglutinating

[9] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 1.

† May, 1945, strains—Chaddick and Ector.

1945-46 strains—Allen, Amdall, Baker, De-Simple, Goodloe, Hacker, Meulder, Mindell, Neubert, Olsen, Orlebeck, Peacock, Potter, Sadowski, Skipton, Solomon.

[10] Francis, T., Jr., Salk, J. E., and Brace, Wm. M., *J. Am. Med. Assn.*, 1946, **131**, 275.

TABLE I.
Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the PR8 Strain of Influenza Virus Type A from 3 Different Lines of Laboratory Passage.

Time at 61.5° (hr)	Previous laboratory passage		
	Ferret No. 198 593 mice 56 eggs	Ferret No. 198 70 mice 719 tiss. cult. 99 eggs	Ferret No. 198 713 mice 3 eggs
0	20,000+	10,000+	10,000+
¼	10,000+	640	0
½	10,000	160+	0
1	2,560	20+	0
2	640+	0	0
4	80+	0	0

TABLE II.
Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the Lee Strain of Influenza Virus Type B from 3 Different Lines of Laboratory Passage.

Time at 61.5° (hr)	Previous laboratory passage		
	8 ferrets 137 mice 101 eggs	8 ferrets 338 mice 8 eggs	13 ferrets 0 mice 7 eggs
0	5,000+	10,000+	2,560
¼	5,000+	5,000+	1,280
½	5,000+	5,000+	80+
1	5,000+	2,560	0
2	5,000+	320+	0
4	5,000+	0	0

activity of the PR8 and Lee strains and found in the course of 4 months at 4 C that the Lee strain was less stable than PR8. The observations described in the present report suggest the likelihood that the different findings in different laboratories may be related to the appearance of true variants, with respect to the property in question, that may have developed in the course of passage of the same original strain.

Jones[11] reported the adaptation to heat of the infectious component of influenza virus induced by subjecting the virus-containing suspension to temperatures of 50 to 56°C between successive passages. In personal communication it has been learned that heat resistance was maintained for 3 passages without heat treatment between passages; longer passage without heating has not been studied. It would appear from Jones' studies that the procedure employed resulted in the same effect reported by Armstrong[12] who was able to select a heat-resistant strain of vaccinia virus by passaging samples of virus that had withstood the longest storage periods at 37°C. In Jones' experiments heat-resistant strains did not develop as a result of passage in embryos incubated at temperatures above the range used normally.

Discussion. Evidence for the occurrence of variation in viruses has recently been reviewed by Stanley[13] and by Burnet.[14] Among the plant viruses variants have been shown to possess differences demonstrable chemically and physically as well as biologically.[13] Among the animal viruses the variations that have been described have been in terms of the biological properties of the viruses. For the

[11] Jones, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 315.

[12] Armstrong, C., *Pub. Health Rep.*, 1929, **44**, 1183.

[13] Stanley, W. M., *Messenger Lectures in Virus Diseases*, Cornell University Press, 1943, 35.

[14] Burnet, F. M., *Virus as Organism*, Harvard University Press, 1945.

influenza viruses it has been shown that different strains of the same type vary in their antigenic makeup[15] as well as in their infective capacities[16] and adaptability for different hosts;[17] strains of virus differ with respect to toxicity[18] and it appears that the capacity for the O to D transformation[19] described by Burnet also varies. Moreover, the characteristics of chicken red cell adsorption-elution, in terms of temperature and time, are different for the PR8 and Lee strains.[1] Studies of certain biophysical properties of a few strains of influenza virus have been made. The biophysical properties which have been considered are related to particle size and electrical charge.[20] With the exception of suggestive variations in size,[21] differences in the properties of strains have not been reported. It would appear reasonable to consider, as another physical property, the resistance to disruption of the biological activities of the virus. From this viewpoint, the present studies have revealed that strains of influenza virus differ widely in at least one biophysical characteristic; namely, the critical temperature of destruction of hemagglutinating activity. It has been found, moreover, that in these terms different lines of the same strain also vary.

In summary it may be said that the absence of uniform reaction in terms of stability to heat, among different strains of virus, and in fact among different lines of the same strain, suggests another approach to the study of the physico-chemical properties of the influenza viruses as well as studies of inheritable variations in these elementary living units.

The practical implications of the present experiments are pertinent to the problem of vaccine antigenicity and stability. The extension of the observations on the stability of the hemagglutinating capacity of the virus to the question of stability of antigenicity is dependent upon the relationship between the hemagglutinating and immunizing properties. Although conclusive evidence of identity, or lack of identity, has not yet been obtained,[2,5] the trend indicates sufficient parallelism to warrant the suggestion that the stability of the hemagglutinin at elevated temperatures may permit predication of stability of immunizing capacity at lower temperatures. Assuming that the same processes occur at the different temperatures, but at different rates, the technic employed in these studies may be of practical value for rapid determination of the manner in which the virus should be manipulated in order to achieve the most stable vaccine. The fact that strains and lines of virus differ in stability suggests the possibility that with strains or lines of similar antigenic valence, the more stable one may be more desirable in a vaccine. While this would appear self-evident, considering the vaccine in the interval between preparation and inoculation, it remains to be seen whether greater resistance to disruption is advantageous after the vaccine has been inoculated. One could speculate either way; *e.g.*, that the more stable virus would be less liable to destruction before it has reached the antibody-producing centers, or that a more stable virus may be less reactive in antibody formation. To what extent these considerations enter the problem of immunization will have to be investigated.

Summary. A report has been made of the observation of variation among different strains and different lines of the same strain of influenza virus with respect to the heat-stability of the hemagglutinating property. Certain theoretical and practical implications of this observation have been discussed.

The technical assistance of Miss Lee Whyte is gratefully acknowledged.

[15] Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

[16] Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.

[17] Francis, T., Jr., *Trans. and Studies of Coll. of Phys. of Phila.*, 1941, **8**, 218.

[18] Henle, W., and Henle, G., *Science*, 1945, **102**, 398.

[19] Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Austral. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 9.

[20] Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 507.

[21] Beard, J. W., Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Feller, A. E., and Dingle, J. H., *Southern Med. J.*, 1944, **37**, 313.