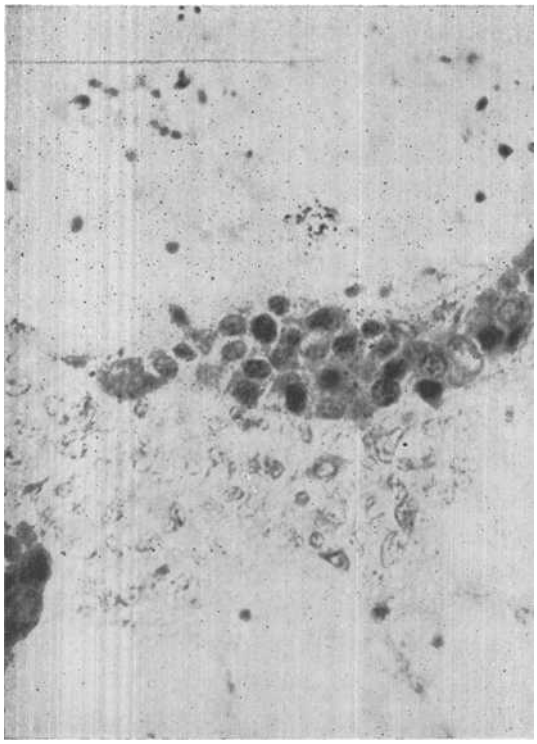

FIG. 3.
Yolk sac, normal uninoculated egg. 18 days' incubation. Nyka's fuchsin-methyl violet stain. × 1050.

in increasing numbers up to the 19th day.

That the bodies are due to staining artefacts seems highly improbable, since they may be demonstrated after different methods of fixation, with different staining techniques, and have a well-defined morphology and sharp outline. Their absence in the yolk cells before the 10th day is a further argument against their being artefacts. There remain two possible interpretations of their nature:

1. They are cytoplasmic constituents of normal yolk cells, appearing in the course of their evolution. Against this viewpoint is the fact that they are not present in all cells and indeed, during the first few days after their appearance, may be scant in number and require prolonged search for their detection.

2. They are elementary virus bodies or psittacosis-like micro-organisms, present as harmless commensals in the developing yolk cells. The finding of diploid forms and the progressive increase in the numbers of the bodies suggests active multiplication. Further experiments are obviously indicated before drawing conclusions as to the nature of these bodies.

Since the submission of this proof, identical bodies have been found in the yolk cells of eggs from Barred Plymouth Rocks and Rhode Island Reds sent to us from the Storrs Agricultural Experiment Station through the courtesy of Dr. Erwin Jungherr.

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Adaptation of Influenza Virus to Heat.*

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Bacteria have been adapted many times to growth or survival under a number of physical conditions, including heat, which are entirely foreign to any environmental circumstances encountered in normal multiplication. Due to numerous technical difficulties, very few similar studies with viruses have been carried out.

Armstrong[1] was able to select a heat-resistant strain of vaccinia virus by passaging samples of virus which withstood the longest storage periods at 37°C. At the end of the 63rd transfer, the virus withstood that temp-

* This investigation was aided in part by the Commission on Influenza Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army, Washington, D.C.

[1] Armstrong, Charles, *Pub. Health Rep.*, 1929, **44**, 1183.

erature for 33 days. Enders and Pearson[2] were unable to cultivate the Melbourne strain of influenza A virus at 41°C, and could not infect day old chicks with that virus, although they could recover virus from their lungs 19 hours after inoculation. They believed that the body temperature of the chick (41°C) might be a limiting factor in their resistance.

A number of investigators (Fairbrother,[3] Henle and Henle,[4] and Hirst[5,6]) have determined the temperatures necessary to inactivate the influenza virus. These workers found that temperatures of 56°C to 57°C for 30 to 45 min were necessary for this inactivation. Determination of virus survival was made by mouse infectivity tests, or by the ability of the virus to initiate growth in chorioallantoic sac of embryonated eggs.

In order to test the adaptability of influenza A virus to heat, 2 strains were used, the mouse-adapted PR8* and the IC† strains of influenza A viruses. The PR8 strain was in its 507th mouse passage, while the IC strain was in its 50th egg passage. The latter strain was isolated in Iowa City during the influenza epidemic of December 1943 by inoculation of throat washings into the chorioallantoic sac of embryonated eggs, and had never been in another medium.

The PR8 strain of influenza virus was studied in relation to its adaptability to propagation at 41°C in tissue culture medium and the chorioallantoic sac of embryonated eggs. Tissue culture medium was prepared in the usual manner under aseptic conditions, using minced 9- to 10-day chick embryo tissue suspended in 4.5 ml of Tyrode's solution placed in 50 ml rubber-stoppered flasks. Such media were stored at 37°C for 1 to 6 days before use. The sterile flasks of tissue culture were inoculated with 0.5 ml of tissue culture fluid containing virus and incubated at 37°C or 41°C for 2 day periods. When storage of infected tissue cultures was necessary, such storage was carried out at refrigerator temperature.

The PR8 strain of influenza A virus grew in tissue culture medium at 41°C, and was carried for 153 passages. Titrations of passages 30 and 91 in mice (Swiss strain) showed active virus present in titers of 10^{-2} for mortality and 10^{-3} for lesion production, as contrasted to titers of 10^{-3} for mortality and 10^{-4} for lesion production with control virus grown at 37°C. This indicated that growth at the higher temperature, although occurring, was less abundant than at 37°C. The virulence for mice apparently remained unchanged, however, because subcultures at 37°C from the 41°C strain gave titers identical with the controls. Continued passage at 41°C did not give evidence of any adaptation to heat, since growth constantly remained poor. The PR8 strain also grew in the chorioallantoic sac (7 passages) of 12-day embryonated eggs incubated at 41°C. Here, also, growth remained poor, as indicated by mouse infectivity tests. One and 2-day-old chicks were given separately the tissue culture or allantoic fluid containing the 41°C and 37°C strains of influenza virus intranasally under ether anaesthesia. Both strains could be recovered from chick lungs 19 to 24 hr after inoculation but not after 48 hr. No evidence was obtained that the 41°C strain survived any longer in the chick than did the control strain.

Since no evidence of adaptation to heat could be obtained by growth of the PR8 strain of influenza virus at 41°C, an inter-transfer method of heat treatment at higher temperatures was adopted to try to increase the resistance of the virus to heat. For preliminary study of heat shock on the virus, the PR8 strain was grown in tissue culture medium incubated at 37°C for 2 days; 1 ml quantities of the virus-containing medium were transferred to cotton-stoppered conical centrifuge tubes or to sealed vials, and were heated at various temperatures in a constant temperature water bath. This heated virus was then inoculated into fresh tissue culture medium and incubated at 37°C. The test for the presence of active

2 Enders, John F., and Pearson, Harold E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 143.

3 Fairbrother, R. W., *Lancet*, 1938, **1**, 1269.

4 Henle, Werner, and Henle, Gertrude, *J. Bact.*, 1943, **46**, 314.

5 Hirst, George K., *J. Exp. Med.*, 1942, **76**, 195.

6 Hirst, George K., *J. Exp. Med.*, 1943, **78**, 88.

* Dr. T. Francis, Jr., supplied the PR8 strain of influenza A virus.

† Dr. Wm. M. Hale supplied the Iowa City strain of influenza A virus.

TABLE I.
Survival of PR8 Strain of Influenza Virus in Tissue Culture Medium After Single Heat Treatment.

Temperature °C	Time min	Survival
60	5	0
	7	0
55	15	+
	30	±*
	45	0
	60	0
50	60	+
	120	+
	180	0

* Occasionally no virus survived.

virus was made by mouse inoculation; all mice received 0.1 ml inocula intranasally under ether anaesthesia. Table I shows the results of single heat treatments. Since parallel results were obtained when the virus was heated in centrifuge tubes and sealed vials, the centrifuge tubes were used thereafter because of their greater convenience.

When the PR8 strain was heated for second treatments at temperatures over 50°C for periods of over 15 min, active virus could not be shown in culture following the second heat treatment. This was not surprising, since Henle and Henle[4,7,8] have shown that inactivated virus exerted a marked inhibitory effect on propagation of active virus. The presence of large amounts of heat-inactivated virus apparently inhibited the growth of whatever small quantities of active virus survived, and the additive effect of a second heating was sufficient to prevent growth entirely. However, by gradually increasing the heat shock period from 15 to 60 min at 50°C between each transfer, the virus was carried for 90 passages. This "adapted" virus was tested for its ability to survive single shock treatment, with results as shown in Table II.

As can be seen from a comparison of the results in Tables I and II, the adapted virus was able to withstand heat shock to a greater degree than the unadapted virus. The adapting temperature, however, could not be increased over 50°C, since at higher temperatures, even for shorter intervals, the virus was repeatedly lost. This was probably caused by the inhibiting effect of a gradually increasing amount of inactive virus.

Further study of adaptive changes in the influenza virus undergoing heat-adaptation was carried out by comparing the effect of storage at different temperatures on this strain and the unadapted control virus. PR8 strain was incubated in tissue culture medium at 37°C for 2 days, then placed unopened at varying storage temperatures. Virus activity was shown by mouse inoculation. All determinations were run in triplicate, and in no case was there appreciable variation in the three determinations. Table III shows the results obtained.

It is evident from Table III that the adapted influenza A virus survived under the various storage temperatures better than did the unadapted control virus.

A subculture of the 80th passage in tissue culture medium of the PR8 strain undergoing heat adaptation at 50°C for 60 min inter-transfer heat treatments was made in the chorioallantoic sac of 12-day embryonated

TABLE II.
Survival of Heat-adapted PR8 Strain of Influenza Virus in Tissue Culture Medium After Single Heat Treatment.

Temperature °C	Time min	Survival
60*	5	+
	7	0
55	15	+
	30	+
	45	±*
	60	0
50	60	+
	120	+
	180	+

* Occasionally no virus survived.

TABLE III.
Survival of Unadapted and Heat-adapted PR8 Influenza Virus in Tissue Culture Medium After Storage.

Temp of storage °C	Survival time-unadapted days	Survival time-adapted days
25.5	28	49
37	18	22
41	8	14

[7] Henle, Werner, and Henle, Gertrude, *Am. J. Med. Sci.*, 1944, **207**, 705.

[8] Henle, Werner, and Henle, Gertrude, *Am. J. Med. Sci.*, 1944, **207**, 717.

eggs. Here the virus withstood the same 50°C heat treatment as in tissue culture, but passage with higher temperature heat shock was not possible.

In order to test further the effect of heat on the PR8 strain a change in procedure was made. Two passages instead of one were allowed between heat shock treatment. The virus-containing allantoic fluid was diluted with equal parts of broth before heat treatment, and the heated virus was adsorbed with a heavy suspension of sterile embryonic chick red blood cells after heating. The red cell-virus mixture was allowed to stand at room temperature until the hemagglutination was marked, and was then used for inoculation of the allantoic sac. It was thought that the inactivated virus would not be as readily eluted as was the active virus, and that this procedure would thereby minimize the inhibiting effect of inactive virus. Heat shock at 56°C for 15 min periods was used for 4 heat treatments, 56°C for 20 min periods for 10 more heat treatments, and finally the virus was able to withstand heat shock of 56°C for 30 min intervals, but was lost on the 20th heating. In several attempts to carry the virus for a greater number of heat shock treatments, the virus died out at a similar stage.

A control of unadapted PR8 strain was subjected to the same procedure, excepting for heat shock. This control and the adapted strain were tested for their ability to withstand single heat shock, with results as shown in Table IV.

Presence of virus was determined by the Rickard[9] modification of Hirst's[5,10] hemagglutination technic. Titrations of the unadapted PR8 strain control usually gave endpoints of 1-200. Titrations of the adapted virus showed only slight, if any, agglutination in the first passages following heat shock treatment, and usually gave endpoints of 1-200 in the second passages. This was probably due to the inhibitive effect of the inactivated virus.

TABLE IV.
Survival of Unadapted and Heat-adapted PR8 Strain of Influenza Virus in Chorioallantoic Fluid After Single Heat Treatment.

Temp °C	Time min	Survival-unadapted	Survival-adapted
60	5	0	0
56	10	+	+
	15	±*	+
	20	0	+
	30	0	+
	45	0	±
	60	0	0

* Occasionally no virus survived.

TABLE V.
Survival of IC Strain of Influenza Virus in Chorioallantoic Fluid After Single Heat Treatment.

Temperature °C	Time min	Survival
60	5	+
	7	0
56	15	+
	20	+
	30	+
	45	0
	60	0

In order to determine whether changes in specificity had occurred during the adaptation to heat, the adapted virus was set up with antiserum to the unadapted control PR8 virus; neutralization occurred.

The heat-adapted and unadapated strains of virus were mixed with a culture of *Eberthella typhosa*, and heated at 56°C for 30 min. The typhoid bacillus and the unadapted virus did not survive this treatment. However, the adapted strain was not inactivated.

A more rapid method of adaptation was attempted using PR8 strain grown in the chorioallantoic sac. Heatings at 56°C for periods increasing by 5 min intervals from 5 to 30 min were used; each heating was followed by chick embryonic red cell adsorption and 2 transfers in the allantoic sac, and each period of time was used twice. The virus became able to survive the two heat treatments at 56°C for 25 but not 30 min periods. This was indication of an increase in ability to withstand heat as compared with the unadapted strain, but was under the limit attained by the slower procedure.

A recently isolated strain of influenza A virus, the IC strain, was included in the study. Preliminary tests of the ability of the IC virus to withstand heat were carried out with virus

[9] Rickard, E. R., Thigpen, Minnie, and Crowley, James H., *J. Immunol.*, 1944, **49**, 263.

[10] Hirst, George K., *J. Exp. Med.*, 1942, **75**, 49.

grown in the chorioallantoic sac of 12-day embryonated eggs. The results are shown in Table V.

Comparison of the initial heat resistance of the PR8 and IC virus strains in Tables IV and V shows a greater ability of the IC virus to withstand heat.

When the IC strain was heated for second treatments at 56°C for 30 min periods, active virus could not be shown in culture following the second heat treatment.

The IC strain was subjected to the more rapid method of adaptation. Heatings at 56°C for periods increasing by 5 min intervals from 10 to 30 min were used; each heating was followed by chick embryonic red cell adsorption and 2 transfers in the allantoic sac, and each period of time was used 4 times. The IC strain became able to survive two heat treatments at 56°C for 30 min periods, but was lost repeatedly on additional heatings. Using the Salk[11] modification of Hirst's hemagglutination technic, which gives agglutinations at greater virus dilutions than the Rickard technic, endpoints were obtained at dilutions of virus of 1-480 in the first passage after heating, and 1-4000 in the second passage. The IC virus strain controls were treated in the same manner, excepting for heat shock, and they also showed titers of 1-4000. Single heat shock tests of the adapted virus surviving the first heat treatment at 56°C for 30 min gave the results seen in Table VI.

TABLE VI.
Survival of Heat-adapted IC Strain of Influenza Virus in Chorioallantoic Fluid After Single Heat Treatment.

Temperature °C	Time min	Survival
60	5	+
	7	0
56	15	+
	20	+
	30	+
	45	+
	60	0

It was evident that this IC strain of virus, which normally had a higher resistance to heat than the PR8 strain, could also be adapted to withstand heat treatment which would normally destroy the unadapted IC virus.

Discussion. Growth of influenza A virus was shown to take place at an elevated temperature (41°C), but no evidence of adaptation of the virus to heat by cultivation at that temperature could be demonstrated.

Influenza virus could be adapted to heat by heat shock treatment between transfers. The procedure which was developed minimizes the antagonistic action of inactivated virus on the growth of active virus by allowing: (1) at least two transfers between each heat treatment, (2) some dilution of the virus-containing fluids, and (3) adsorption of virus with fowl red blood cells. A technic such as this was particularly necessary when temperatures and duration of treatment approximated very closely the inactivation conditions for unadapted virus. The presence of heat-inactivated virus exerted a marked inhibitory effect on the propagation of active virus, and repeatedly exerted a cumulative effect so that successive transfers under a particular condition became impossible. It may be possible to increase the ability of the influenza virus to withstand even greater temperatures for longer periods of time by long-continued transfers with very gradually increasing heat shock.

Conclusions. 1. Two strains of influenza A virus were trained to withstand temperature conditions which were normally lethal. 2. Influenza A virus could not be adapted to heat by growth at elevated temperatures.

[11] Salk, Jonas E., *J. Immunol.*, 1944, **49**, 87.