TABLE I.
Effect of Zinc Ion on Serum Alkaline Phosphatase Activity in Patients With and Without Cancer.

Group	No. of cases	Ratio of phosphatase activity in presence of zinc to that in its absence		
		Range	Mean	Stand. dev.†
Individuals without cancer*				
a. At 10^{-5} M Zn^{++}	25	0.87-1.12	1.01	0.056
b. At 10^{-4} M "	14	0.81-1.04	0.89	0.066
c. At 10^{-3} M "	16	0.42-0.60	0.50	0.055
Patients with cancer				
d. At 10^{-5} M Zn^{++}	39	0.94-1.07	1.00	0.025
e. At 10^{-4} M "	22	0.77-1.00	0.90	0.058
f. At 10^{-3} M "	24	0.27-0.60	0.45	0.079

* This group consisted of normal individuals and patients with miscellaneous diseases, chiefly heart disease. We are indebted to Dr. Harry Gold for the use of the latter patients from the Beth Israel Hospital Cardiac Clinic.

† The values for the standard deviation were used in calculating "P" values. The "P" values for the comparison of groups *a* with *d*, *b* with *e*, and *c* with *f* were, respectively, 0.4, 0.6, and 0.04. The "P" values for the comparison of groups *a* with *b*, *a* with *c*, *d* with *e*, and *d* with *f* were all less than 0.01.

values represented significant changes. Thus, in patients with cancer, as in those without, 10^{-5} M Zn^{++} had no effect on the serum alkaline phosphatase activity, whereas 10^{-4} and 10^{-3} M Zn^{++} produced significant decreases.

Statistical treatment showed that there was no significant difference between the mean values for the ratios of the individuals without cancer and the patients with cancer at any of the 3 concentrations of Zn^{++}. In view of the high "P" values for these differences, it was considered very unlikely that the extension of the study to larger numbers of patients would bring the "P" value down to 0.01 and render the differences significant.

Summary. A concentration of 10^{-5} M Zn^{++} failed to affect the serum alkaline phosphatase activity in individuals either with or without cancer. These results are not in agreement with the findings of Roche *et al.*[1,2] that this concentration of Zn^{++} activates the serum alkaline phosphatase activity of individuals without cancer and inhibits this activity in patients with cancer. Concentrations of 10^{-4} and 10^{-3} M decreased the serum alkaline phosphatase activity, but the extent of these decreases was the same in patients with and without malignancy.

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Multiplication of Influenza Virus in Dead Chick Embryos.

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Recently, the cultivation of *Rickettsia prowazeki* in dead chick embryos was described by Rabinowitz, *et al.*[1] These workers showed that the rickettsiae employed multiplied abundantly in embryos which, although dead, still contained surviving cells. They confirmed the earlier work of Bucciante[2] and demonstrated that dead embryos may contain living cells for surprisingly long periods. After holding 3-day-old embryos at 4°C for 24

1 Rabinowitz, E., Aschner, M., and Grossowicz, N., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 469.

2 Bucciante, L., *Arch. exp. Zellforsch.*, 1931, **11**, 397.

hours, then at room temperature for 7 days, and finally at 37°C for 16 days, they were able to culture living cells from the embryo or its membrane in plasma clots.

It seemed of both practical and theoretical interest to determine whether influenza virus would multiply in dead chick embryos. A number of experiments were carried out in which the time and temperature, at which embryos were held, were varied over wide ranges. The results obtained indicate that the PR8 strain of influenza A virus is capable of multiplication in the tissues of dead chick embryos if incubation at 35°C is sufficiently prolonged. They show also that the virus multiplies in dead embryos to an extent comparable to its multiplication in the living embryo.

Materials and Methods. The PR8 strain of influenza A virus was employed. It was cultivated in the allantoic sac of 9-day embryos which were incubated at 39°C before inoculation and at 35°C thereafter. Infected allantoic fluid gave hemagglutination and embryo infectivity titers of the order of 1:2000 and $10^{-8.5}$, respectively. Infected fluid was diluted in sterile nutrient broth; 0.2 cc of either a 10^{-3} or 10^{-6} dilution was employed as the inoculum.

The embryos of White Leghorn eggs were used. They were incubated at 39°C for periods ranging from 5-10 days. In certain experiments, after inoculation of the virus into the allantoic sac, embryos were held at room temperature for 3-10 days, and thereafter incubated at 35°C for 2-10 days. In other experiments, normal embryos were chilled at 4°C for either 20 or 96 hours, some of the former then held at room temperature for 3 days, after which they were inoculated into the allantoic sac with virus. Following this, the embryos were held at room temperature for an additional period of 2-10 days, and finally incubated at 35°C for 2-10 days. Groups of 2-3 embryos were employed and a variety of different experimental conditions were used. Specimens of allantoic fluid were removed repeatedly from individual embryos, usually at intervals of 2 days. In this way it was possible to follow the multiplication of the virus in individual embryos.

Measurement of the concentration of virus in the extra-embryonic fluid was carried out by the hemagglutination technique as described previously.[3] Washed chicken RBC in a final concentration of 0.25% were used. The allantoic fluid dilution-RBC mixtures were held at room temperature for one hour. The titration end point was taken as that dilution which gave a 2+ reaction. Hemagglutination-inhibition tests[3] with specific immune rabbit sera were carried out with a constant dilution of serum capable of inhibiting hemagglutination by 128 units of the homologous virus.

Virus infectivity titrations were carried out as described previously.[4] Serial tenfold dilutions of fluid were inoculated into the allantoic sac of 9-day chick embryos and, after incubation at 35°C for 48 hours, the allantoic fluids were removed and tested by the hemagglutination procedure. The infectivity end point was calculated in the usual way.

Results. It was found that the time required to kill embryos at room temperature varied somewhat with their age. Thus, 5-day embryos held at room temperature for one day were almost always dead; 8-day embryos held for 2 days were usually dead, and invariably when held for 4 days; 10-day embryos held for 3 days were usually dead, and invariably when held for 4 days. Only 5-day embryos were held at 4°C and in every instance it was found that 20 hours at this temperature resulted in death. Embryos were considered to be dead when spontaneous movements had ceased, blood vessels showed no pulsation and no further development occurred.

The results obtained with embryos held for long periods at room temperature after inoculation with the PR8 strain are shown in Table I. It was found that multiplication of the virus did not occur when embryos were kept at room temperature for periods up to 8 days and not incubated at 35°C thereafter. However, with inoculated embryos which had been held at room temperature for 7-10 days, and then incubated at 35°C for periods ranging from 2-10 days, definite evidence of mul-

[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[4] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

TABLE I.
Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at Room Temperature for Long Periods After Inoculation.

Age of embryos (days)	Inoculum 0.2 cc intra-allantoic (dilution)	After inoculation: Room temp. 22-25°C (days)	After inoculation: 35°C (days)	No. embryos	Allantoic fluid: Hemagglut. titer*	Allantoic fluid: Embryo-infectivity titer
5	10^{-3}	3-8	—	3	0,0,0	
7	,,	,,	—	,,	0,0,0	
9	,,	,,	—	,,	0,0,0	
5	,,	7	2	2	8 16	
,,	,,	,,	4	,,	128,128	
,,	,,	,,	8	,,	256,256	$>10^{-5}$
8	,,	,,	2	,,	0,0	
,,	,,	,,	4	,,	0,128	
,,	,,	,,	8	,,	512,2048	$>10^{-5}$
10	,,	,,	2	,,	0,0	
,,	,,	,,	4	,,	0.0	
,,	,,	,,	8	,,	0.32	
5	,,	10	3	,,	0 32	
,,	,,	,,	5	,,	8,256	
,,	,,	,,	7	,,	512,2048	$>10^{-5}$
8	,,	,,	3	,,	0 0	
,,	,,	,,	5	,,	0,32	
,,	,,	,,	7	,,	C,128	$>10^{-5}$
5	10^{-6}	7	2	3	0,0,128	
,,	,,	,,	6	,,	C,1024,1024	
,,	,,	,,	10	,,	C,2048,2048	
5	,,	10	3-10	,,	0,0,0	

* Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

tiplication of the virus was obtained. In contrast to what occurs in the allantoic sac of living embryos,[5] maximum hemagglutination titers were not obtained with dead embryos until the period of incubation at 35°C had been extended to 6 or 8 days. In certain instances hemagglutination titers as high as 1:2000 and embryo infectivity titers of at least 10^{-5} were obtained with fluid from dead embryos. Hemagglutination-inhibition tests with immune sera served to identify the virus; complete inhibition was demonstrated repeatedly with anti-PR8 serum, while anti-Lee serum did not inhibit the reaction. Even when the inoculum was as small as 10^{-6}, *i.e.* approximately 10^2 E.I.D., multiplication of the virus occurred. It is noteworthy that inoculated embryos which were held for 10 days at room temperature were capable of supporting the multiplication of influenza virus when they were incubated at 35°C. It should be reiterated that, irrespective of the age of the embryos employed, none was capable of surviving for longer than 4 days at room temperature.

The results obtained with embryos which were killed by chilling at 4°C, and held for various periods at room temperature before inoculation with the PR8 strain, are shown in Table II. In these experiments all embryos employed were 5 days of age. In the large majority of instances embryos which had been inoculated after death were capable of supporting the multiplication of influenza virus if they were incubated at 35°C. Moreover, dead embryos held at room temperature for 3 days before inoculation or 4 days there-

[5] Pearson, H. E., *J. Bact.*, 1944, **48**, 369.

TABLE II.
Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at 4°C or at Room Temperature for Long Periods Before Inoculation.

Before inoculation			After inoculation			
4°C (hr)	Room temp. 22-25°C (days)	Inoculum 0.2 cc intra-allantoic (dilution)	Room temp. 22-25°C (days)	35°C (days)	No. embryos*	Allantoic fluid hemagglut. titer†
20	—	10^{-3}	4	2	3	64,64,64
,,	—	,,	,,	6	,,	512,256,256
,,	—	,,	,,	10	,,	128,128,64
,,	—	,,	9	2-6	,,	0,0,0
,,	—	10^{-6}	4	2	,,	0,0,0
,,	—	,,	,,	4	,,	C,2048,64
,,	—	,,	,,	10	,,	C,2048,2048
,,	—	,,	7	3-9	,,	C,0,C
,,	—	,,	10	2-5	,,	C,0,C
—	4	10^{-3}	4	3	,,	256,0,64
—	,,	,,	,,	6	,,	32,1024,128
—	,,	,,	8	3	2	64,0
—	,,	,,	,,	7	,,	16,C
20	3	,,	4	3	3	64,0,0
,,	,,	,,	,,	6	,,	512,C,0
,,	,,	,,	,,	10	,,	512,C,C
,,	,,	,,	8	3-7	,,	0,0,0
96	—	,,	—	2	,,	0,0,8
,,	—	,,	—	5	,,	512,512,1024
,,	—	,,	—	11	,,	2048,2048,2048
,,	—	,,	2	3-9	,,	0,0,0
,,	—	,,	4	3-10	,,	0,C,C
,,	—	,,	8	3-7	,,	0,0,0

* All embryos were 5 days of age when inoculated.
† Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

after supported viral multiplication. Maximum hemagglutination titers comparable to those obtained with live embryos were demonstrated with dead embryos when incubation at 35°C was carried out for 4-6 days. Quantities as small as 10^2 E.I.D. of virus were capable of initiating multiplication in such embryos. It is of interest that even when embryos were held at 4°C for as long as 96 hours, they were still capable of supporting multiplication of the virus. It should be emphasized that such embryos had been dead for at least 3 days before inoculation.

In other experiments embryos were frozen at –30°C and held for 20 hours. They were then thawed, inoculated with a 10^{-3} dilution of virus and incubated at 35°C for prolonged periods. Under these conditions no evidence of multiplication was obtained in any instance.

The extra-embryonic fluid obtained from dead embryos had a gross appearance very different from that which is withdrawn from live embryos. In most instances the fluid was cloudy or turbid and frequently it contained some yolk, albumin, or both. As might be expected, hemagglutination reactions obtained with such fluids were somewhat abnormal, especially in the lower dilutions. However, in the higher dilutions hemagglutination appeared to be nearly normal and the end point was usually determined easily.

Discussion. Multiplication of influenza virus in the tissues of dead chick embryos is probably analogous to its multiplication in

chick embryo tissue culture medium. It was surprising, however, to find that when dead embryos were incubated at 35°C for a sufficient period the virus titer became much higher than is found in liquid tissue culture.[6] The evidence obtained does not suggest that the virus is capable of multiplying in the absence of living cells. It has been demonstrated clearly[1,2] that in embryos killed by chilling at 4°C or by prolonged storage at room temperature, living cells are present. Presumably these cells carry on their usual metabolic functions when held at 35°C. It seems probable that the long period of incubation necessary for the development of maximal virus titers may be related to and dependent upon the multiplication of such living cells in both the dead embryo and its membranes. In this connection it will be recalled that when embryos were frozen at –30°C they were thereafter incapable of supporting viral multiplication.

It is apparent that these results may be of practical usefulness, especially in field work where attempts are made to recover viruses from infected persons or animals. In the case of influenza virus, at least, it seems evident that it is not necessary to observe special precautions with chick embryos. When adequate laboratory facilities are lacking, dead embryos which have been stored for considerable periods may prove satisfactory for the recovery of the infectious agent.

Summary. Chick embryos killed by prolonged storage at room temperature or 4°C are capable of supporting the multiplication of influenza virus upon prolonged incubation at 35°C.

6 Weller, T. H., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 124.

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Relation of Vitamin B_{12} to Liver Basophilia.*

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Vitamin B_{12} has been reported to stimulate the growth of chicks on a vegetable protein diet and of certain lactobacilli.[1,2] When it was later found that the nucleoside thymidine can replace vitamin B_{12} in the medium of these microorganisms, it was postulated that the vitamin acts as an enzyme in the synthesis of nucleosides and thus of nucleotides and nucleic acids.[3,4] It was decided to test this theory by studying the formation of nucleic acids in the rat as influenced by vitamin B_{12}. Since it is generally accepted by histochemists that the basophilia reaction determines ribonucleoprotein,[5] the effect of vitamin B_{12} on the basophilia of rat livers was studied in this experiment.

Experimental. Weanling white rats whose mothers had been fed a diet in which the sole source of protein was soybean oil meal were used in this investigation. When the young were 21 days old they were removed from the mothers and divided into 3 groups. Groups 1 and 2 received a diet of the purified type in which soybean oil meal was the sole source

* Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Agricultural Biochemistry.

1 Ott, W. H., Rickes, E. L., and Wood, T. R., *J. B. C.*, 1948, **174**, 1047.

2 Shorb, Mary L., *Science*, 1948, **107**, 397.

3 Wright, L. D., Skeggs, Helen R., and Huff, J. W., *J. B. C.*, 1948, **175**, 475.

4 Shive, W., Ravel, Joanne M., and Eakin, B. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

5 Demsey, E. W., and Wislocki, G. B., *Phys. Rev.*, 1948, **26**, 1.