the colonies of the mutant were much smaller when plated in the presence of large numbers of parent cells.

Summary and conclusions. The results indicate that *B. abortus* loses its requirement for added CO_2 by spontaneous mutation at a rate of approximately 3×10^{-10} per cell division. Training of such cultures to dispense with their added CO_2 requirement probably consists of a selection of a spontaneously occurring mutant rather than gradual adaptation of the culture as a whole. The low mutation rate indicates a high degree of stability for CO_2 requirement in cultures of *B. abortus*.

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The Effect of Tocopheryl Esters on Influenza Virus. (18225)

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Miller and Dessert(1) have found that tocopheryl esters are capable of inhibiting the enzymatic depolymerization of hyaluronic acid by hyaluronidase, *in vitro*. Chemically there are certain similarities between hyaluronic acid (tissue mucin), glandular mucin and ovomucin. The latter two have been shown to behave as a substrate for the influenza virus enzyme. By competing in this capacity with the receptor substance of the red cell they prevent or inhibit* hemagglutination(2-5). The hemagglutination preventing property of ovomucin is gradually destroyed in the presence of live influenza virus, but is not affected by virus that has been heated 56°C for 30 minutes (in the case of PR 8). One type of hyaluronidase (pneumococcal) is also capable of altering ovomucin so that it no longer prevents hemagglutination by heated influenza virus(3).

The present paper undertakes to show that tocopheryl esters will also inhibit the enzymatic activity of influenza virus *in vitro*, and in addition are capable of lowering slightly the infectivity of the virus for chick embryos when the embryos are pre-treated with tocopheryl esters.

Materials and Methods. The PR 8 strain of influenza virus was used. The virus was propagated in 10-12-day-old chick embryos and stored in the form of frozen allantoic fluid. Ovomucin was prepared essentially by the method used by Gottschalk and Lind(5) The tocopheryl acid esters studied were the disodium salts of dl-alpha-tocopheryl phosphate (dl-alpha-Tph),† d-alpha-tocopheryl phosphate (d-alpha-Tph),‡ d-alpha-tocopheryl succinate (d-alpha-TS),‡ and a mixture of approximately equal concentrations of d-gamma-tocopheryl and d-delta-tocopheryl phosphates, referred to as d-delta, gamma-Tph.‡ Alpha-tocopherol itself was not studied because of the difficulty in obtaining a suitable solution. The tocopheryl esters were sparingly soluble in the 0.01 M phosphate buffer at pH 7.4, containing 0.85 per cent NaCl used as solvent and diluent for all reagents except

* To avoid confusion the action of ovomucin on hemagglutination commonly called inhibition will, in this paper, be called prevention, and the term inhibition will be reserved for the action of the tocopheryls.

1. Miller, W. H., and Dessert, Alice M., *Ann. N. Y. Acad. Sciences*, 1949, v52, 167.

2. Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, v160, 404.

3. Hardy, P. H., and Horsfall, F. L., *J. Exp. Med.*, 1948, v88, 463.

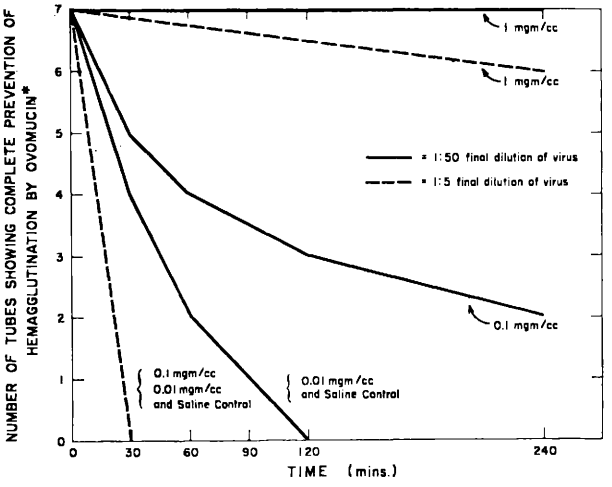
4. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 442.

5. Gottschalk, A., and Lind, Patricia E., *Brit. J. Exp. Path.*, 1949, v30, 85.

† Hoffmann-LaRoche. A synethetic product.

‡ Distillation Products, Inc. Isolated from natural sources.

THE EFFECTIVENESS OF DIFFERENT CONCENTRATIONS OF dl-α-TPh AGAINST TWO DILUTIONS OF VIRUS
FIG. I (See Table I)



* It will be noted that by plotting the number of tubes instead of the actual dilution the ordinate is on a logarithmic scale. The other figures are also plotted this way.

where otherwise noted. The resulting solutions were close to neutrality.

In vitro tests. The amount of ovomucin present in a given solution was estimated in terms of the highest dilution at which the solution prevented hemagglutination by a given amount of heated PR 8 virus. Virus was added to a cold solution of ovomucin to which had been added either saline or a tocopheryl ester. The mixture was placed in a 37°C water bath. At appropriate intervals the tubes were shaken, samples withdrawn and immediately heated in boiling water for two minutes to destroy the virus present and stop the reaction. This amount of heating did not affect the ovomucin. To determine the residual amount of unchanged ovomucin, the sample was cooled and diluted serially 2-fold in saline (after an initial 1:10 dilution) in volumes of 0.4 cc. Eight to 16 hemagglutinating doses (HD) of virus previously heated to 56°C for 30 minutes were added in a volume of 0.2 cc. After 30 minutes at room temperature 0.2 cc of 0.5% suspension of thrice-washed chicken red blood cells (RBC) not more than five days old were added. The tubes were allowed to sit undisturbed at room temperature at least one-hour and then checked by the pattern technic(6) for hemagglutination prevention due to ovomucin. All experiments included a saline and ovomucin control (without virus) and a chicken cell agglutination (CCA) titer on the heated indicator virus dilution used. Tocopheryl esters in the concentrations used in these experiments did not affect the CCA titer of heated or unheated virus when the tocopheryl was added to the allantoic fluid (AF) and the fluid then diluted. In higher concentrations they caused hemolysis. When added to ovomucin solutions the tocopheryls caused an increased opalescence, but this did not affect the titration of the hemagglutination preventing property.

In vivo tests. The following procedure was used for the infectivity determinations: The required number of living 10-day-old chick embryos were inoculated via the allantoic cavity with the tocopheryl esters in various amounts, contained in volumes of 0.1-0.5 cc. An equal number of embryos were given an equal volume of buffered saline. By way of "sterilizing" the tocopheryl, penicillin was added to it and also in equal concentration to the saline inoculum so that each egg received about 250 units.§ Both sets of embryos (treated and saline controls) were then inoculated intraallantoically with 0.1 or 0.2 cc

6. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

TABLE I. Effect of Various Concentrations of DL-alpha-Tph on Ability of PR8 to Destroy Hemagglutination-prevention Properties of Ovomucin. Ovomucin, 3 cc.

Vol. of reagents in order of addition			Ovomucin hemagglutination-prevention titer									
			Tube No.									
			1	2	3	4	5	6	7	8	9	
		Time	Dilution									
1 cc of DL-alpha-Tph	1 cc of PR8	(min.)	10	20	40	80	160	320	640	1280	2560	C*
1 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0†
		240	0	0	0	0	0	0	±	+	+	0†
0.1 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
0.01 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
0 (1 cc saline)	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
1 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0†
		240	0	0	0	0	0	0	0	+	+	0†
0.1 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	0	±	+	+	+	0
		60	0	0	0	0	±	+	+	+	+	0
		120	0	0	0	+	+	+	+	+	+	0
		240	0	0	+	+	+	+	+	+	+	0
0.01 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	±	+	+	+	+	0
		60	0	0	±	+	+	+	+	+	+	0
		120	+	+	+	+	+	+	+	+	+	0
0 (1 cc saline)	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	±	+	+	+	+	0
		60	0	0	±	+	+	+	+	+	+	0
		120	+	+	+	+	+	+	+	+	+	0

* C = Control—no indicator virus.
† Slight hemolysis.
16 HD per tube.
+ = Hemagglutination.
0 = No hemagglutination.
The figure in the DL-alpha-Tph and PR8 columns represent final concentration and final dilution, respectively.

of the same serial dilution of a stock pool of PR 8 (allantoic fluid) stored frozen in small amounts in stoppered vials. Four to five dilutions were inoculated straddling the $I.D._{50}$ endpoint, using eight eggs per dilution per set. The shell holes were sealed with paraffin. The eggs were then placed in a 35° incubator, candled at 24 hours for viability and at 48 hours removed to a cold room (4°C) overnight. The allantoic fluids were harvested and each fluid tested for hemagglutination by mixing equal volumes of 0.25% RBC and AF. Hemagglutination was taken to indicate infection. The $I.D._{50}$ endpoints were calculated by the method of Reed and Muench(7). The allantoic fluids from the treated embryos often showed a noticeable opalescence. This was present in both the infected and non-infected embryos and was found to be considerably less or completely absent when fluids were harvested without previous chilling of the embryo.

Results. In Fig. 1 and Table I are presented the results of an experiment employing various concentrations of dl-alpha-Tph, two concentrations of virus and a constant amount of ovomucin. The undiluted allantoic fluid had a CCA titer of 1:2560. In this experiment the 10^{-1} dilution was made with the same virus-containing allantoic fluid heated 65°C for 30 minutes to destroy the virus. This was done to make certain that the effects obtained when the virus was diluted were due to virus dilution alone and not to dilution of some non-specific (*e.g.* mineral) factor in the AF. When 1 cc of undilute AF was added to the

§ When streptomycin (calcium salt) was mixed with the tocopheryl a heavy precipitate formed. It was not used even though there were indications that the tocopheryl was still active when injected.

7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

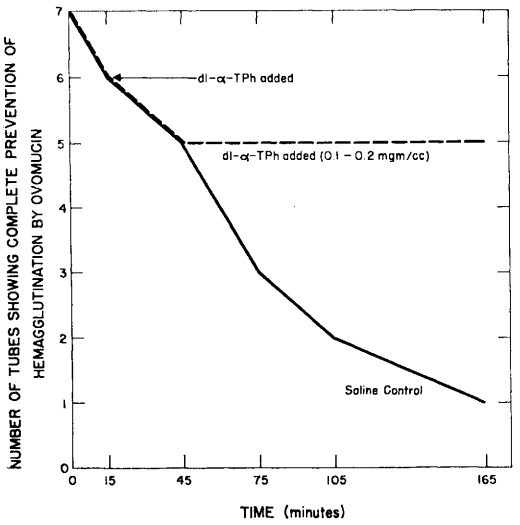
ovomucin-tocopheryl the final dilution became 1:5, and similarly for 10^{-1} the final dilution became 1:50. It can be seen that with the substrate (ovomucin) constant the degree of inhibition by tocopheryl was directly related to the amount of tocopheryl present and inversely related to the amount of virus.

Corollary experiments varying the concentration of substrate were not done. Increasing the ovomucin concentration (without concentrating the virus) necessitated prolonged incubation periods which would have allowed too much opportunity for non-specific factors to come into play. Decreasing the substrate concentration by a significant amount made for insufficient spread between the initial and final titrations.

It was then determined whether the tocopheryl ester could exert its effect after the virus-substrate reaction had already been initiated. Virus and ovomucin were mixed and placed in a 37°C water bath. Samples were taken at 0 time and at 15 minutes. Then the virus-ovomucin mixture was divided equally among three tubes containing respectively saline and dl-alpha-Tph in sufficient quantity to make final concentrations of 0.1 and 0.2 mg per cc. In the control tube ovomucin continued to be destroyed, whereas in the tubes containing dl-alpha-Tph the ovomucin titer fell at first then leveled off. Fig. 2 illustrates these results. The 4 tocopheryl esters that were available were then compared. All were tested at a concentration of 0.1 mg per cc, and were always run simultaneously against various concentrations of virus. Fig. 3 gives the results with 1:50 final dilution of virus. Dilutions of 1:25, 1:37.5, 1:62.5 and 1:75 gave the same relative results; d-alpha-Tph and dl-alpha-Tph were most effective, d-alpha-TS somewhat less so, and d-delta, gamma-Tph hardly at all.

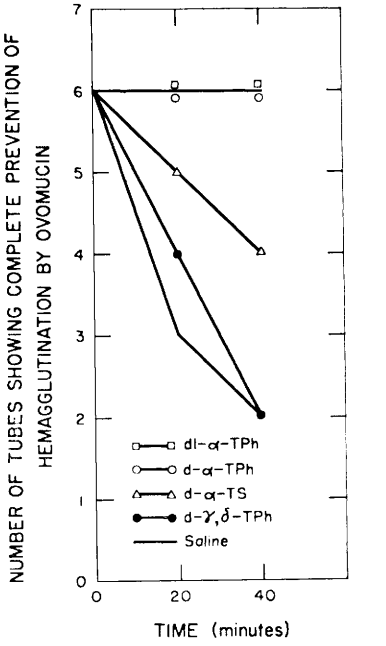
EFFECT OF ADDITION OF dl-αTPh AFTER PR8–OVOMUCIN REACTION HAS BEGUN

FIG. II



RELATIVE EFFECTIVENESS OF VARIOUS TOCOPHERYLS AGAINST 1:50 VIRUS

FIG. III



The following experiments were devised to determine whether the action of the tocopheryl esters was reversible or whether it induced a permanent change in the virus that persisted when the virus was removed from the esters. Allantoic fluid containing PR 8 was treated with dl-alpha-Tph and the virus was then precipitated by cold methanol and recovered according to the method of Cox, *et al.*(8). An untreated control was similarly manipulated.

The addition of dl-alpha-Tph to the un-

8. Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, v56, 149.

TABLE II.
Chick Embryo Infectivity Experiments.
A.

Exp. No.	Treatment	mg per egg	Log $L.D._{50}$
E-10	Saline	—	8.3
	DL-alpha-Tph	1	7.5
11	Saline	—	8.4
	DL-alpha-Tph	1	7.6
	DL-alpha-TS	1	7.5
13	Saline	—	8.3
	D-alpha-Tph	1	7.7
	D-delta, gamma-Tph	1	8.1

B.
10^{-8} Dilution

Controls		Treated	
Positive by h*	Negative by h*	Positive by h*	Negative by h*
21	11	9	23

*h = Hemagglutination.

diluted AF to a final concentration of 1 mg per cc did not cause a noticeable decrease in viral activity. The virus recovered by precipitation was also active. When the concentration of dl-alpha-Tph was increased to 2 mg per cc the viral enzyme activity was inhibited both before and after recovery by precipitation and there was also a decrease in recoverable virus. These experiments, however, were considered inconclusive.

Chick embryo infectivity experiments: Table II-A gives a summary of 3 infectivity experiments performed as outlined above, using a dose of 1 mg per egg of tocopheryl. Table II-B represents the combined results at the 10^{-8} dilution for the 3 experiments (not including d-delta, gamma-Tph). When evaluated by the Chi squared method using the Yates modification for small numbers and by "Student's" "T test" these differences are significant. In those treated eggs in which infection occurred, the CCA titer was equal to that of the controls, the virus was active in breaking down ovomucin and on further passage caused infection. Further passage of some of the fluids from the treated, non-infected embryos did not cause infection at dilutions of 10^{-0} and 10^{-3}. In experiments employing doses of 0.5 mg per egg the effect was smaller. Doses of 2 mg per egg were toxic.

It will be noted that d-delta, gamma-Tph had little if any effect. This corresponds with the *in vitro* findings. The relative effectiveness of these compounds *in vitro* and *in vivo* against influenza virus are the same as found by Miller and Dessert(1) against hyaluronidase *in vitro*.

Discussion. The possible modes of action of the tocopheryl esters upon influenza virus are many and for a discussion of the action of these compounds upon other enzyme systems, the reader is referred to(9), especially Part II. The experiments reported herein, however, appear to indicate some specificity for the alpha configuration against influenza virus enzyme. It is not immediately apparent why, if the activity of the tocopheryl esters against *in vitro* enzyme systems are due to the protein precipitant or detergent-like or metal-ion combining properties conferred upon them by the phosphate radical, the same activity is not exhibited by the phosphate esters of delta and gamma tocopherol. This same question was raised by Miller and Dessert(1) with regard to hyaluronidase. The other enzyme systems discussed in(9) appear to be inhibited equally as well by the delta and gamma tocopheryl esters as by the alpha configuration.

It would seem worth while to prepare other tocopheryl esters and examine their activity against influenza virus.

Summary. Alpha-tocopheryl esters are capable of inhibiting the enzymatic activity of influenza virus *in vitro* and of protecting chick embryos against infection by small doses of virus. A mixture of delta and gamma tocopheryl esters was not effective.

9. Symposium on Vitamin E., *Ann. N. Y. Acad. Sciences,* 1949, v52, 63.

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