No increase in resistance has as yet been demonstrated by exposure of organisms to increasing concentrations of DHSM and PAS used together. These experiments are being continued.

Discussion. While it is recognized that *in vitro* results cannot necessarily be interpreted as *in vivo* effects, the *in vitro* difference between the results when a chemical compound (DHSM-PAS) was used as opposed to when the 2 drugs were added separately, make careful *in vivo* studies important. It had been hoped that the small amount of PAS which could be introduced into the streptomycin molecule might be sufficient to increase the effectiveness of the latter, as well as to reduce the important drawback of the rapid emergence of resistance. These experiments indicate that drug resistant organisms do emerge readily when a strain of tubercle bacilli is exposed to such a compound and that these organisms are resistant not only to the compound itself, but to both streptomycin and PAS as well.

Summary. 1. The *in vitro* growth of the H37Rv strain of tubercle bacilli was inhibited by 0.82 μg DHSM/cc, by a concentration of DHSM-PAS containing 0.41 μg DHSM/cc and by 0.205 μg DHSM/cc when PAS was added in a 3:1 molecular ratio of PAS:DHSM. 2. The fifth serial transfer of H37Rv into increasing concentrations of DHSM resulted in a strain growing readily in over 2000 μg DHSM/cc while the tenth such transfer of the organisms into increasing concentrations of DHSM-PAS resulted in a strain growing readily in over 1000 μg DHSM-PAS/cc. No increase in resistance on the part of organisms transferred into increasing concentrations of DHSM with PAS present in a 1:3 molecular ratio has so far been found. 3. Organisms resistant to the compound (DHSM-PAS) were found to be also resistant to DHSM alone, to PAS alone, and to the mixture of DHSM and PAS.

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Genetic Aspects of the Added Carbon Dioxide Requirements of *Brucella abortus.** (18224)

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It is well known that many strains of *Brucella abortus* require approximately 3 to 10% CO_2 in air for growth on primary isolation(1). However, some isolates lose the requirement for added CO_2 on continued subculture and may then be grown in air alone. The stability of the added CO_2 requirement of 9 strains of *B. abortus* was investigated as an initial step in the study of the role of CO_2 in the metabolism of members of this species.

Methods and cultures. Albimi brucella broth and agar were used as routine culture media and 1 ppm crystal violet was added to the agar for use in making plate counts. The diluent was an aqueous solution of 0.2% Difco tryptose-0.5% NaCl. CO_2 incubations were carried out in vacuum desiccators and the desired pCO_2 obtained by partial evacuation and replacement with tank CO_2. All incubations were at 38°C. Total counts were made using a Quebec colony counter and mutant counts were made using a dissecting microscope with transmitted light. Cultures

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1. Wilson, G. S., *Brit. J. Exp. Path.*, 1931, v12, 88.

TABLE I. Occurrence of Mutants Capable of Growth in Air in Cultures of *Brucella abortus* Krause.

Series	No. of cultures	No. of mutants	Cultures with no mutants	Total cells per culture
1	10	32	0	1.5×10^9
2	10	31	1	1.7×10^9
3	11	121	4	1.6×10^9
4	19	37	6	1.1×10^9
5	20	8	12	1.1×10^9
6	1	11	—	1.0×10^{10}

TABLE II. History and Mutation Rate for the Loss of Added CO_2 Requirement for Several Strains of *B. abortus*.

Strain	Source	Mutations per cell division $\times 10^{-10}$
Krause	Laboratory of Hygiene; human isolation	6.4
96251	Same	4.3
6232C	Camp Detrick; CO_2 stable	1.5
47257	Laboratory of Hygiene; human isolation	2.7
45095	Same	2.3
57087	Same	2.7
V-1	Univ. of Texas; guinea pig passage strain	3.2
335	Univ. of Texas; bovine udder isolation	3.9
1-SHL	Univ. of Texas; human isolation	3.2

were purified by single colony isolation. The sources of the strains employed in this investigation are listed in Table II.

Experimental. For the determination of mutation rate, flasks containing 100 ml broth were inoculated with 10^4 to 10^5 cells from a 24-hour culture grown under 10% CO_2. One ml of the inoculated broth was then transferred aseptically to each of 15 sterile (1.5 x 9 cm) tubes or 10 ml to each of 10 sterile Pyrex milk dilution bottles and incubated in a 10% CO_2-90% air mixture for 48 hours. Samples were taken from 2 or 3 cultures of each series for total counts which were determined by dilution and plating. These plates were incubated for 4 days in 10% CO_2. Mutant counts were determined by direct plating of whole 1 ml cultures or aliquots of the larger 10 ml cultures. These plates were incubated for 4 days in air. Results of a typical experiment are given in Table I. In each experiment several colonies from the plates incubated in air were transferred to agar slants. The mutants, which no longer require added CO_2 for growth, did not revert on serial subculture in either air or 10% CO_2. Cultures were tested for purity by Gram staining and high titer agglutination in antiserum against *B. abortus*. Both parent and mutant strains were smooth.

From the type of data given in Table I mutation rates could be calculated from the total number of mutants or from the fraction of cultures containing no mutants using the formulas of Luria and Delbrück(2) as modified by Lederberg(3). The data in Table II indicate very similar mutation rates for the 9 strains tested. The mean rate for this mutation is approximately 3×10^{-10} per cell division. A much greater variation in mutant counts was found in individual cultures than in replicate samples from the same culture, which indicates that the mutation is spontaneous and is not induced by the lower pCO_2 of air.

Calculation of mutation rate from the total number of mutants depends on similar growth rates of mutant and parent in mixed culture where the ratio of mutant to parent is very small. To test growth rates under these conditions a flask of broth was inoculated with the parent Krause stock and a mutant strain derived from it. The initial ratio of mutant to parent was $1.0:10^7$. After 48 hours incubation in 10% CO_2 the ratio was still $1.0:10^7$ although total counts had increased 10-fold. Growth rates of the two strains in pure culture when incubated in 10% CO_2 were identical during the logarithmic phase of growth.

It appeared possible that not all of the mutants were capable of forming colonies in the presence of large numbers of nonproliferating parent cells. To test this possibility a mutant strain from the parent Krause stock was plated in agar containing a background of 10^8 cells per ml of the Krause strain and in agar medium alone. The plates were then incubated in air. There was no significant difference in the counts of the two series, but

2. Luria, S. E., and Delbrück, M., *Genetics*, 1943, v28, 491.

3. Lederberg, J., personal communication.

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.